UNIVERSIDAD COMPLUTENSE DE MADRID
FACULTAD DE VETERINARIA
Departamento de Sanidad Animal

TESIS DOCTORAL

Toxoplasmosis y neosporosis en rumiantes domésticos: normalización de modelos animales y evaluación de nuevos fármacos

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR

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Madrid
Ed. electrónica 2019

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D. Roberto Sánchez Sánchez

Madrid, septiembre de 2018

COMPLUTENSE UNIVERSITY OF MADRID

VETERINARY FACULTY

Animal Health Department

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DOCTORAL THESIS

D. Roberto Sánchez Sánchez

Madrid, September 2018

Memoria presentada por D. Roberto Sánchez Sánchez para optar al grado de Doctor por la Universidad Complutense de Madrid

Madrid, 19 de septiembre de 2018

La realización de esta tesis doctoral ha sido posible gracias a la financiación del Ministerio de Educación, Cultura y Deporte (MECD) del Gobierno de España, mediante un contrato de Formación de Profesorado Universitario (FPU) FPU/03438, de la cual he sido beneficiario.

La financiación de las investigaciones ha sido posible gracias a los siguientes proyectos:

- *Ministerio de Economía y Competitividad (MINECO) del Gobierno de España (AGL2016-75935-C2-1-R).*
- *Plataforma Tecnólogica de Sanidad Animal de la Comunidad de Madrid (PLATESA) (S2013/ABI2906).*
- *Instituto de Salud (National Institutes of Health, NIH) y Departamento de Agricultura de Estados Unidos (United States Department of Agriculture, USDA) (grants 2014-67015-22106, R01 AI111341 and R01 HD 080670).*

D. Ignacio Ferre Pérez, Doctor en Veterinaria y Profesor titular en el Departamento de Sanidad Animal de la Facultad de Veterinaria de la Universidad Complutense de Madrid; **Dña. Michela Re**, Doctora en Veterinaria y Profesora contratada Doctor en el Departamento de Medicina y Cirugía Animal de la Facultad de Veterinaria de la Universidad Complutense de Madrid, y **D. Luis Miguel Ortega Mora**, Doctor en Veterinaria y Catedrático de Universidad adscrito al Departamento de Sanidad Animal de la Facultad de Veterinaria de la Universidad Complutense de Madrid

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Que la tesis doctoral titulada "Toxoplasmosis y neosporosis en rumiantes domésticos: normalización de modelos animales y evaluación de nuevos fármacos" que presenta el Licenciado en Veterinaria D. **Roberto Sánchez Sánchez** ha sido realizada en las dependencias del Departamento de Sanidad Animal de la Facultad de Veterinaria, de la Universidad Complutense de Madrid bajo su supervisión y cumple todas las condiciones exigidas para optar al grado de Doctor por la Universidad Complutense de Madrid con Mención Internacional.

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2) Los apartados de resumen, introducción, resultados, discusión y conclusiones de la tesis doctoral han sido redactados en una en una de las lenguas habituales para la comunicación científica en su campo de conocimiento, distinta a cualquiera de las lenguas oficiales en España (inglés).

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AGRADECIMIENTOS

"El que da nunca debe volver a acordarse; pero el que recibe nunca debe olvidar".

En primer lugar me gustaría dedicar las primeras líneas a mi familia que desde muy pequeño me fue enseñando lo que es trabajar con animales. A pesar de los madrugones, y los malos momentos cuanto tengo que agradecer a mi padre **Félix** por todo lo que me ha enseñado y me sigues enseñando "anda que no te queda por aprender". Tú nunca me quisiste comprar ovejas para la finca pero tranquilo como ves aquí ya he tenido bastantes. Sin todo lo que tú me has enseñado esto no hubiera sido posible. A mi madre **Lola** que siempre decía ¡pero hijo cuantas horas echas en el laboratorio! pues te aseguro que esto no ha sido fácil, pero con esfuerzo todo es posible. A mi hermana **Sara** que aunque lo suyo ya se veía venir que no eran los animales siempre ha estado hay para lo que me ha hecho falta.

Como no dar las gracias a mis directores, jefes o como queramos llamarlos. A **Ignacio**, por tener siempre abierta la puerta del despacho a cualquier pregunta más o menos importante, por todos esos "cables" que me has hecho los findes con las ovejas y por haber confiado en mi. A **Luis**, por ser un ejemplo de cómo se debe investigar y trabajar, y por sus valiosos comentarios y sugerencias en cada momento. En un principio yo iba a hacer cosas con vacas, así empecé, pero que poquito me duro, al poco me metiste un rebaño de ovejas y de esas de no he salido. A **Micky** por toda tu ayuda que ha sido enorme, todas esas ecografías a cualquier hora. No nos cansábamos de repetir, late late con ese ecógrafo maravilloso que "vale mas que tu vida".

Gracias a toda esa familia de Saluvet, sin vosotros esto no hubiera sido posible. Al Black Friday os remito. A **Alex** por acudir siempre en mi rescate en los momentos más dificiles, también quedan buenos momentos con el cachopo en ka la Mery. **Laura Jiménez** por toda tu ayuda con las ovejas y por salvarme la vida en el incendio del apar, sin tin ahora mismo sería ceniza. También por hacerme ver en tantas ocasiones la realidad de las cosas. **Carlos**, que aunque lo suyo son los terneros siempre estuvo disponible y le tocó lo peorcito con las ovejas. **Marta**, que has sido quien aún en mis peores momentos he seguido gastandola bromas a pesar de saber que me la estaba jugando. A **Pilar** por estar siempre dispuesta a ayudar aunque ello te lleve a caerte todo lo larga que eres. A **Patri** Eskerrik asko por todo, apareciste en el grupo en mi momento rebaño y tuvimos que acabar llamando a emergencias el primer dia "Oso ondo". A **Lola**, simplemente por aguantarme, que ya es mucho. A **Jorge** y **Luis Corpa**, "gemeliers" gracias por todo, a vosotros también os tocó pringar como internos. A las nuevas generaciones saluvetianas, a **Rafa**, por decirte algo de lo poco que tienes bueno que eres del Real Madrid, a **Merche** por hacerme un hueco en el animalario y soportar mis ovinos, a **Raquel** por ser un ejemplo del trabajo bien hecho. A **Laura Rico**, porque si tú puedes podemos todos, llegarás lejos. A **Ali**, parte de lo que hay en esta tesis doctoral fue debido a tu ayuda con los cultivos. A **Angela**, por tu sencillez, aunque siempre me haces preguntas muy difíciles. A **David**, desembarcaste hace poco en Saluvet pero me has sufrido bastante, gracias por dejarme que te robara el ordenador y por esa ayuda de clínico especialista con los corderos.

Gema, **Esther** y la más rubia de todo el departamento (**Vane**), muchísimas gracias por hacer que todo funcione en el laboratorio. A **Javier Regidor**, por resolverme tandas dudas y darme tu apoyo en todo momento, compartimos grandes momentos en Uruguay. A **Mercedes** por ser tan cercana y por toda la confianza que transmite. A **Javier Blanco** por hacer que las instalaciones del Hospital no se hayan mantenido sino que se hayan mejorado poco a poco por tu empeño. A **Pizarro**, **Marta** y **Tabanera** por estar siempre disponibles, habéis sido fundamentales en esta tesis. Gracias a **Cinta**, **Javier**, **Chema** e **Isabel** por estar ahí, siempre he podido contar con vuestra ayuda.

A todos aquellos que ya no están en el laboratorio pero con los que he compartido momentos inolvidables. A **Paula**, que te voy a contar que no sepas de lo que ha sido esta tesis, también es verdad que poco a poco hemos ido mejorando y ya al menos no planeamos partos para el dia de nochevieja. A **David**, eres un gran tio "finocchio", gracias por todo lo que me has enseñado, eres un gran investigador, me gustaría en el futuro poder trabajar contigo de nuevo. A **Iván** por ser un ejemplo de superación día a día, estoy orgulloso de ti por enseñarnos que fuera de España también hay vida. Al **Moreno**, no hace tanto que marchaste "pájaro" pero aun no me he asumido que ya no estés en el grupo, mil gracias por tu ayuda con los ratones, los gatos y con las ovejas en los primeros momentos. A **Silvia**, por tener tanta paciencia, por enseñarme mis primeras nociones de búsqueda bibliográfica y por acogerme siempre en Asturias.

A todos los visitantes de fuera que estuvieron en el laboratorio: A los argentinos, **Marcelo**, **Yanina** y **Nacho** por vuestro salero argentino, por compartir tantas cosas juntos. A los brasileños, **Muller**, **Pomy**, **Junior** y **Wagnner** por vuestra compañía y amistad. A los mejicanos **Lety**, **Adrian**, **Carlos Cruz** y **Cristina Guerrero** por ser tan buena gente y enseñarnos tanto. A los italianos, **Luca** y **Alessia** siempre tan cercanos. A **Carol**, por tu alegría, por tu asombrosa capacidad de adaptación y fortaleza.

No me puedo olvidar de los pilares de los experimentos. Todos los Residentes que han pasado por el servicio de rumiantes del Hospital, **Lydia**, **Isa**, **Lucas**, **Lola**, **Natalia, Verónica, Neus y Victor** que han colaborado siempre con todo y su ayuda ha sido fundamental para que todo saliera bien junto con los **internos de rumiantes** que siempre han mostrado un enorme interés por esta investigación. En especial a **Irene** que sintió curiosidad por las ovejas y siguió colaborando sin descanso. También agradecir a **Ignacio Alvarez**, **Isabel** y **Pedro** por su ayuda en las cirugías que tan complicadas eran. Agradecer a **Ito**, **Juanfran**, **Jacobo** y **Juanjo** por el excelente cuidado que siempre han dado a las ovejas. Tambien agradecer a **Nacho** y **Ramón** por tener abierta siempre la otra puerta de la sala por lo que pudiera hacer falta. También agradecer a **Fidel** por su inestimable ayuda con todo el material necesario para los muestreos. Gracias a **Gustavo** por esas analíticas tan detalladas con tanta rapidez.

Durante estos años he tenido la gran suerte de poder trabajar en diferentes laboratorios, conociendo excelentes personas que de un modo u otro, merecen un agradecimiento. A todas las personas del IGM en León donde se han hecho tantas cosas relacionadas con esta tesis doctoral. En especial a **Julio** y a **Dani** por su inestimable ayuda. También a todos los que me sufrieron en Serida de Asturias, en especial a **Ali** y a **Koldo**. Gracias toda la gente de la Universidad de Zaragoza por ser la base de la pirámide que ha sustentado esta tesis doctoral, a **Luis Miguel Ferrer, Luis Miguel Cebrian**, **Chema**, **Juanjo**, **Delia**, **Fran**, **Teresa**, **Calasanz** y sus todos los **alumnos colaboradores.** *I also want to acknowledge all the effort made from the United States by* ***Wes***, ***KK***, ***Lynn***, ***Matt, Kasey*** *and* ***Ryan*** *among others, although the beginning with teleconferences without understanding anything was complicate. I also thank people in Bern. Thank you* ***Andrew***, ***Bruno***, ***Joachim***, ***Norbert***, ***Walter***, ***Britta***, ***Vreni***, ***Pablo***, ***Nicoleta***, ***Junhua***, ***Dominic***, ***Anika***, ***Reto***, ***Ghalia***, ***Rita*** *and* ***Adriana*** *for making me have an incredible stay there.*

Me gustaría hacer un agradecimiento especial a la persona que más me ha apoyado y que me ha dado fuerzas para poder terminar esta tesis, gran parte de esta tesis es también tuya **Cris**, gracias por todo. También a **Lolita** y a **Victor** por comprenderme y apoyarme en todo lo que han podido. También me acuerdo mucho de aquellas personas que comenzaron conmigo esta tesis doctoral y que por desgracia hoy ya no están con nosotros, gracias **abuela Manola** por cuidarme tanto siempre, **abuelo Félix** por ayudarme a conservar entre generaciones la pasión por los animales, **tio Abilio** "Amezua,

como ves aquí con todas las ovejas que hemos utilizado aquí habíamos formado un rebaño" y **tia Maxi** por como me querias, esta tesis va por vosotros.

Por último dedico estas últimas palabras a mis fieles amigos que siempre han dado todo el apoyo para que esta tesis pudiera salir adelante, os debo una.

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Seguridad y eficacia frente a la infección por *T. gondii* del BKI-1294 en ovejas gestantes

Seguridad y eficacia frente a la infección por *N. caninum* del BKI-1553 en ovejas gestantes

APPENDIX I LITERATURE REVIEW

LISTADO DE ABREVIATURAS

°C	Grados centígrados	Grades centigrade
ADN/DNA	Ácido desoxirribonucleico	Deoxyribonucleic acid
ALP	Fosfatasa alcalina	Alkaline phosphatase
ASF	Tamaño medio del foco	Average size focus
AST	Aspartato transaminasa	Aspartate aminotransferase
AUC	Area bajo la curva	Area Under the Curve
ATP	Adenosín trifosfato	Adenosine triphosphate
BAG	Antígeno de bradizoíto	Bradyzoite antigen
BKI	Inhibidores de la proteina kinasa	Bumped kinase inhibitor
bp	Pares de bases	Base pairs
BVD	Virus de la enfermedad de la frontera	Border Disease Virus
CBC	Recuento sanguíneo completo	Complete blood count
CDPK	Proteina quinasa dependiente de calcio	Calcium dependent protein kinase
CK	Creatina quinasa	Creatin kinasa
cm^2	Centímetro cuadrado	Square centimetre
Cmax	Concentración máxima	Maximum concentration
CNS	Sistema nervioso central	Central nervous system
CO_2	Dióxido de carbono	Carbon dioxide
ConA	Concanavalina A	Concanavalin A
dg	Días de gestación	Days of gestation
dpi	Días post-infección	Days post-infection
DHFR	Dihidrofolato reductasa	Dihydrofolate Reductase
DHPS	Dihidropteroato sintetasa	Dihydropteroate synthase
DNA	Ácido desoxirribonucleico	Deoxyribonucleic acid
EDTA	Acido etilendiaminotetraacético	Ethylenediaminetetraacetic acid
e.g.	*Exempli gratia* (por ejemplo)	*Exempli gratia* (for instance)
ELISA	Ensayo inmunoenzimático	Enzyme-linked immunosorbent assay
ELQ	Quinolonas tipo endochin	Endochin like quinolones
FCS	Suero fetal bovino	Fetal calf serum
FL	Líquido fetal	Foetal liquid
g	gramo	gram
GGT	Gamma-glutamil transferasa	Gamma-glutamyl transferase
GRA	Antígeno de gránulos densos	Dense granule antigen
h	horas	hours
HE	Hematoxilina y eosina	Haematoxylin and eosin
hERG	Gen relacionada con los canales de potasio	Human ether-a-go-go-related gene
HFF	Fibroblastos de piel de prepucio humana	Human Foreskin fibroblasts
HP	Histopatología	Hispathology
IC_{50}	Concentración inhibitoria 50	Inhibitory concentration 50

i.e.	*Id est* (esto es)	*Id est* (this is)
IFAT	Inmunofluorescencia indirecta	Indirect immunofluorescence assay
IFN-γ	Interferón gamma	Gamma Interferon
IgG	Inmunoglobulina G	Immunoglobulin G
IL	Interleuquina	Interleukin
IM	Intramuscular	Intramuscular
IP	Intraperitoneal	Intraperitoneal
IRG	GTPasa relacionda con sistema inmune	immunity-related GTPase
IV	Intravenoso/a	Intravenous
kg	Kilogramo	Kilogram
LCMS/MS	Cromatografía liquida y espectometría de masas	Liquid chromatography‑tandem mass spectrometry
LD_{50}	Dosis letal 50	Lethal dose 50
MAG	Proteína de la matriz quística	Cyst matrix protein
MAT	Test de microaglutinación	Microagglutination test
MIC	Proteína de micronemas	Microneme protein
mg	Miligramo	miligram
min	Minuto	Minute
ml	Mililitro	Millilitre
mM	Mili molar	Milli molar
mm	Milímetro	Millimetre
mm^2	Milímetro cuadrado	Square millimetre
n	Número de muestra	Sample number
NA	No aplicable	Not applicable
Nc	*Neospora caninum* (prefijo)	*Neospora caninum* (prefix)
NK	(Células) asesinas naturales	Natural killer (cells)
nM	Nanomolar	Nanomolar
NRC	Consejo Nacional de Investigación	National Research Council
OD	Densidad óptica	Optical density
OR	Odds ratio	Odds Ratio
PBS	Tampón fosfato salino	Phosphate Buffer Saline
PBMC	Célula mononuclear de sangre periférica	Peripheral blood mononuclear cell
PCR	Reacción en cadena de la polimerasa	Polymerase chain reaction
PEG	Polietilenglicol	Polyethylene glicol
pi	Post-infección	Post-infection
PK	Farmacocinetica	Pharmacokinetics
PMSG	Gonadotropina coriónico de yegua gestante	Pregnant mare's serum gonadotropin
PO	“Per os” administración oral	“Per os” oral administration
pp	Postparto	Postpartum
qPCR	PCR cuantitativa	Quantitative PCR

RBC	Recuento de células de la serie roja	Red blood cell count
RFLP	Polimorfismos de longitud de fragmentos de restricción	Restriction Fragment Length Polymorphism
RIPC	Índice relativo por cien	Relative index per cent
ROP	Proteína de cuerpo de roptrias	Rhoptry bulb protein
RT	Temperatura ambiente	Room temperature
SAG	Antígeno de superficie	Surface antigen
SBV	Virus Schmallenberg	Schmallenberg virus
SC	Subcutánea	Subcutaneous
SD	Desviación estándar	Standard deviation
SNP	Polimorfismo de nucleótido único	Single-nucleotide polymorphism
SRS	Secuencia SAG1 relacionada	SAG1-related sequence
Tg	*Toxoplasma gondii* (prefijo)	*Toxoplasma gondii* (prefix)
TLR	Receptor tipo *toll*	*Toll*-like Receptor
TNF-α	Factor de necrosis tumoral alpha	Tumour necrosis factor alpha
TY	Recolección de taquizoitos	Tachyzoite yield
UE/EU	Unión europea	European union
UK	Reino Unido	United Kingdom
UPLC	Cromatografía de alta resolución en fase líquida	Ultra performance liquid chromatography
US	Ultrasonografía	Ultrasonography
USA	Estados Unidos de América	United States of America
UV	Ultravioleta	Ultraviolet
WBC	Recuento de células sanguíneas de la serie blanca	White blood cell count
WB	Western blot	Western blot
μm^2	Micrometros cuadrados	Square micrometers
µM	Micromol	Micromol
µg	Microgramo (10^{-6} gramos)	Micrograms (10^{-6} grams)
µl	Microlitro (10^{-6} litros)	Microliter (10^{-6} litres)
X^2	Chi cuadrado	Chi square

CAPÍTULO I

RESUMEN/SUMMARY

Toxoplasma gondii y *Neospora caninum* son parásitos apicomplejos muy relacionados filogenéticamente y considerados como unas de las principales causas de aborto infeccioso en pequeños rumiantes y ganado vacuno respectivamente en todo el mundo. Sin embargo, recientemente, la importancia de *N. caninum* como agente causante de abortos en ganado ovino se ha incrementado enormemente. *T. gondii* en pequeños rumiantes y *N. caninum* en ganado vacuno presentan una elevada prevalencia y producen importantes pérdidas económicas como consecuencia del fallo reproductivo. *T. gondii* en ganado ovino produce principalmente aborto tras una primoinfección con ooquistes durante la gestación mientras que *N. caninum* puede producir abortos tras una infección durante la gestación o tras la recrudescencia de una infección crónica. Para el control de la toxoplasmosis en ganado ovino existe una vacuna viva atenuada comercializada (Toxovax™), la cual reduce parcialmente los abortos asociados a *T. gondii*, mientras que la la única vacuna comercializada hasta el momento para la neosporosis en ganado vacuno (Neoguard), basada en taquizoitos muertos, se ha retirado del mercado debido a su baja eficacia. En relación al tratamiento, se han probado diferentes clases de fármacos tales como macrólidos, poliéteres ionóforos, inhibidores de la síntesis del folato, quinolonas y triazinas y han mostrado eficacia moderada. Por lo tanto, hasta el momento, no hay fármacos disponibles para el tratamiento de la toxoplasmosis y la neosporosis en rumiantes. Asi pues, el desarrollo de medidas de control es de gran importancia.

Los modelos *in vitro* representan una alternativa para optimizar el uso de animales, pero los modelos *in vivo* son la mejor opción para ensayos terapéuticos y vacunales asi como para estudiar la patogenia, la respuesta inmune y las consecuencias de la infección. El modelo murino es frecuentemente utilizado para determinar la virulencia de *T. gondii* y para estudiar la toxoplasmosis congénita. Sin embargo, no se conoce si las diferencias en placentación, fisiología reproductiva y respuesta inmune entre el ratón y los rumiantes podrían conllevar unas consecuencias diferentes tras la infección durante la gestación. Aunque varios aislados tipo II de *T. gondii*, el más prevalente en todos los hospedadores en Europa incluyendo la oveja, han sido evaluados en ovejas gestantes, ninguno de estos aislados se ha estudiado profundamente en ratón.

En la neosporosis, el interés por el modelo ovino gestante se ha incrementado en los últimos años ya que representa una alternativa viable al costoso modelo bovino y al modelo murino que es escasamente extrapolable al bovino. Sin embargo, la influencia de la dosis de infección y la ruta de administración en el resultado de la infección apenas se ha estudiado.

Para intentar progresar en el control de la toxoplasmosis y neosporosis, se han evaluado *in vitro* y en modelos de animales de laboratorio grupos farmacológicos tales como tiazolidas, diamidinas, artemisininas, naftoquinonas, agentes anticancerígenos, "endochin-like quinolones" y inhibidores de la proteína quinasa dependiente de calcio tipo 1 (CDPK1), mostrando unos resultados prometedores. La CDPK1 es una buena diana terapéutica ya que está ausente en células de hospedadores mamíferos, pero conservada entre los parásitos apicomplejos y esencial para numerosas etapas en el ciclo lítico del parásito. Esta CDPK1 puede ser inhibida por unos compuestos competidores por el sitio de unión del ATP de la CDPK1 llamados "BKIs", los cuales son eficaces frente a *T. gondii* y *N. caninum in vitro* y en modelos murinos gestantes. Sin embargo, la seguridad y eficacia de los BKIs en ovejas gestantes y su eficacia en modelos ovinos gestantes de toxoplasmosis y neosporosis no se conoce.

Con estos antecedentes, el **objetivo general** de la presente Tesis Doctoral fue estandarizar modelos murinos y ovinos gestantes de infección por *T. gondii* y comparar la virulencia de aislados tipo II de *T. gondii* entre ratón y oveja, estandarizar un modelo ovino gestante para el estudio de la infección por *N. caninum* y evaluar la seguridad y eficacia frente a *T. gondii* y *N. caninum* de los BKIs en ovejas gestantes.

En el **primer objetivo específico**, el fenotipo *in vitro*, la virulencia en ratón y la infección congénita en ratón y oveja de un aislado tipo II de *T. gondii* recientemente obtenido (TgShSp1) fueron comparadas con el aislado de laboratorio tipo II de referencia (TgME49). Un mayor crecimiento y menor formación de quistes *in vitro* y una mayor mortalidad de las crías y transmisión vertical tras la infección de ratones gestantes con el aislado de laboratorio TgME49 en comparación con el aislado TgShSp1 asi como una DL_{50} de 10^3 taquizoitos en ratones para el aislado TgME49 sugieren un incremento en la virulencia del aislado TgME49 probablemente debido al alto número de pases en cultivo o ratón. Por lo tanto, parece razonable especular que los resultados de los estudios que utilizan aislados de laboratorio deberían ser validados con aislados de pase bajo antes de extrapolar las características generales de un determinado tipo. La presencia de signos clínicos leves, sin mortalidad en ratones no gestantes y gestantes inoculados por vía intraperitoneal con taquizoitos (1-10^5 taquizoitos) o por vía oral con ooquistes (25-2000 ooquistes) del aislado de tipo II TgShSp1 sugiere una virulencia muy baja en ratones adultos para este aislado.

En ovejas infectadas por vía oral con ooquistes, no se observaron diferencias entre estos dos aislados en la mortalidad perinatal o en las lesiones o número de corderos positivos a *T. gondii*. La infección congénita del aislado TgShSp1, evaluada mediante la mortalidad perinatal y la transmisión vertical fue diferente en función del hospedador. En ratones, se observó mortalidad en el 50% de las crías cuando una ratona fue desafiada con una dosis alta de ooquistes (500 ooquistes del aislado TgShSp1) mientras que en las ovejas inoculadas con la misma dosis de ooquistes, todos los fetos murieron (100%). Asimismo, se observó mortalidad en el 9% y 27% de las crias tras la infección en ratones con 100 y 25 ooquistes del aislado TgShSp1, respectivamente, mientras que en ovejas, la infección con 50 y 10 ooquistes del aislado TgShSp1 produjo mortalidad en el 68% y 66% de los fetos/corderos. Tan solo se observaron diferencias en la transmisión vertical en crias que sobreviven tras la infección de ratones y ovejas con una dosis baja de ooquistes (100% tras la infección de ovejas con 10 ooquistes del aislado TgShSp1 y 37% en ratones tras la infección con 25 ooquistes del aislado TgShSp1). En conclusión, la virulencia en ratón de los aislados tipo II de *T. gondii* quizás no sea un buen indicador para predecir las consecuencias de las infección en ovejas gestantes.

En el **segundo objetivo específico**, se investigó la clínica, la respuesta inmune, la detección y carga del parásito y la gravedad de las lesiones en tejidos placentarios y cerebro fetal tras el desafío por vía intravenosa de ovejas a mitad de gestación con 10^5, 10^4, 10^3 ó 10^2 taquizoitos o por vía subcutánea con 10^4 taquizoitos del aislado virulento Nc-Spain7. Todas las ovejas gestantes inoculadas con 10^5 taquizoitos abortaron, mostrando además un incremento más temprano de la temperatura rectal y de los niveles de IFNγ y una mayor respuesta inmune humoral y carga parasitaria en el cerebro fetal en comparación con aquellas ovejas desafiadas por vía intravenosa con dosis más bajas. Incluso la infección por vía intravenosa con la dosis más baja (10^2 taquizoitos) produjo mortalidad fetal en el 50% de las ovejas y 100% de transmisión vertical, sin diferencias en la carga parasitaria comparando con la infección intravenosa con 10^5 taquizoitos. Por otro lado, las ovejas gestantes desafiadas por vía subcutánea con 10^4 taquizoitos mostraron menor incremento de las temperaturas rectales y niveles de IFNγ comparadas con las ovejas desafiadas por vía intravenosa con la misma dosis de taquizoitos. Sin embargo, no se observaron diferencias dependientes de la vía de administración en la respuesta inmune humoral, la mortalidad fetal ni en la trasmisión vertical. Estos resultados sugieren que una dosis superior a 10^5 taquizoitos por vía intravenosa es un desafío excesivamente agresivo para ovejas que podría ocasionar el descarte de fármacos o vacunas potencialmente eficaces. Esa dosis, 10 veces inferior a la que se había testado previamente, y esa vía de administración parecen ser suficientes para ocasionar 100% de aborto. En base a estos resultados se propone el uso de un modelo basado en la inoculación de una dosis máxima de 10^5 taquizoitos por vía intravenosa en futuros experimentos, para así obtener resultados más fiables y realistas.

En el **tercer objetivo específico**, las ovejas gestantes se dosificaron por vía oral con el BKI-1294 (5 dosis de 100 mg/kg cada 48 horas) o por vía subcutánea con el BKI-1553 (2 pautas de dosificación: 1ª dosis de 35 mg/kg y una semana después una 2ª dosis de 10 mg/kg ó 7 dosis de 10 mg/kg cada 48 horas). La eficacia de estos fármacos fue evaluada tras la administración a las 48 horas después de la infección con ooquistes del aislado TgShSp1 por vía oral (BKI-1294) con taquizoitos del aislado Nc-Spain7 por vía intravenosa (BKI-1553). Además, se determinaron las concentraciones de fármaco en las ovejas y sus fetos (solo para el BKI-1553). Las ovejas gestantes dosificadas por vía subcutánea con el BKI-1553 o por vía oral con el BKI-1294 presentaron concentraciones terapéuticas de fármaco en plasma, con concentraciones máximas de 11 μM y 2 μM y mínimas de 4 μM y 0.4 μM para el BKI-1553 y el BKI-1294 respectivamente. Asimismo, el BKI-1553 fue capaz de cruzar la barrera placentaria encontrándose concentraciones terapéuticas de 1 μM en el plasma fetal. Sin embargo, el BKI-1553 y de forma más acusada el BKI-1294 mostraron un rápido aclaramiento plasmático. El BKI-1294 y el BKI-1553 parecen ser seguros ya que no se encontraron alteraciones en la temperatura rectal ni en los parámetros hematológicos y bioquímicos ni en la gestación ni daño local asociado con estos fármacos. Sin embargo sí que se encontraron diferencias en seguridad en función de la vía de administración. Mientras que la dosificación oral no incrementó la temperatura rectal ni modificó la consistencia fecal, la administración subcutánea desencadenó la aparición de nódulos dérmicos asociados a un incremento de temperatura rectal y monocitosis.

La administración del BKI-1294 a ovejas infectadas con *T. gondii* y del BKI-1553 a ovejas infectadas con *N. caninum* se asoció a un menor incremento de las temperaturas rectales, por lo tanto a una menor replicación del parásito. Los estudios *in vitro* han evidenciado la presencia de complejos intracelulares multinucleados asociados al tratamiento con el BKI-1294 y BKI-1553 de cultivos celulares infectados con *T. gondii* y *N. caninum*, con una sobreexpresión del antígeno de taquizoito SAG1 y el marcador de bradizoito BAG1. Estos complejos multinucleados, compuestos por pre-zoitos incapaces de separarse y formar taquizoitos podrían influir en la respuesta inmune celular y humoral frente a *T. gondii* y *N. caninum* ya que la administración de estos fármacos desencadenó un incremento en la producción de IFNγ y la presencia de anticuerpos anti-*T. gondii* SAG1. En modelos ovinos gestantes de infección por *T. gondii* y *N. caninum* en los cuales todas las ovejas sin tratar abortaron, el tratamiento con el BKI-1294 en ovejas infectadas con *T. gondii* evitó la mortalidad en el 76% de los fetos y el tratamiento con el BKI-1553 en ovejas infectadas con *N. caninum* protegió del aborto al 37-50% de las ovejas. En relación a la transmisión vertical, mientras que el BKI-1553 no evitó la transmisión vertical de *N. caninum*, aunque disminuyo parcialmente los efectos de la infección reduciendo las lesiones y la presencia del parásito y la carga en cerebro fetal, el BKI-1294 fue capaz de prevenir la transmisión vertical de *T. gondii* en el 53% de los corderos.

En definitiva, en esta Tesis Doctroal se ha profundizado en la comparación entre el ratón y la oveja de la virulencia de aislados tipo II de *T. gondii*, se ha estandarizado un modelo ovino gestante de infección por *N. caninum* y se ha evaluado la seguridad de los BKIs y su eficacia en frente a *T. gondii* y *N. caninum* en modelos ovinos gestantes.

Toxoplasma gondii and *Neospora caninum* are apicomplexan parasites very related phylogenetically and considered one of the main causes of infectious abortion in small ruminants and cattle respectively worldwide. However, recently, the importance of *N. caninum* as causative agent of abortion in sheep has greatly increased. *T. gondii* in small ruminants and *N. caninum* in cattle show high prevalence and produce important economic losses as a consequence of the reproductive failure. *T. gondii* in sheep mainly induces abortion triggered by primoinfection with oocysts during pregnancy, while *N. caninum* related abortions can be found after infection during pregnancy or recrudescence. For the control of toxoplasmosis in sheep there is a commercial attenuated live vaccine available (Toxovax™), which partially reduced *T. gondii* related abortions. No vaccine is available in the market for the control of neosporosis in cattle, since the only vaccine commercialised so far (Neoguard), based on killed tachyzoites, was withdrawn from the market on account of the low efficacy shown. Concerning treatment, different drug classes such as macrolide antibiotics, polyether ionophore antibiotics, folate inhibitors, quinolones and triazinones have been evaluated in ruminants showing moderate efficacy. Therefore, to date, no drug is available for the treatment of toxoplasmosis and neosporosis in ruminants and the improvement and development of control measures is of paramount importance.

In vitro models represent an alternative to optimize the use of animals, but *in vivo* models represent the best option to carry out therapeutic or vaccine trials, as well as to study pathogenesis, immune responses and outcomes of infection. Mice model is often used to determine the virulence of *T. gondii* isolates and to study congenital toxoplasmosis. However, it is remains as an open question if differences on placentation, reproductive physiology and immune responses between mice and ruminants could trigger a differential outcome of infection during pregnancy. While several *T. gondii* type II isolates, the most prevalent lineage in all hosts in Europe including sheep, have been evaluated in pregnant sheep, none of this isolates have been studied deeply in mice.

In neosporosis, the interest of pregnant sheep models has increased in the last years as a valid alternative to the costly bovine model and to the poorly comparable murine model. However, little is known concerning the outcome of infection with differerent infectious doses or routes of administration.

In an attempt to progress in the control of toxoplasmosis and neosporosis, drug classes such as thiazolides, diamidines, artemisinins, naphthoquinones, anticancer agents, endochin-like quinolones and calcium-dependent protein kinase have been evaluated *in vitro* and in small animal models showing highly promising results. Calcium dependent protein kinase 1 (CDPK1) represents a promising drug target, as it is absent in mammalian hosts, but conserved among apicomplexan parasites and essential for several processes in the lytic cycle of the parasite. CDPK1 can be effectively targeted by a class of ATP-competitive compounds named bumped kinase inhibitors (BKIs), which were effective against *T. gondii* and *N. caninum in vitro* and in pregnant mice models. However, the safety of BKI compounds in pregnant sheep and their efficacy in pregnant sheep models of toxoplasmosis and neosporosis are unknown.

With this background, the **general objective** of the present Doctoral Thesis was standardize pregnant mice and sheep models of *T. gondii* infection and compare the virulence of *T. gondii* type II isolates between mice and sheep, standardize a pregnant sheep model for the study of *N. caninum* infection, and evaluate the safety and efficacy against *T. gondii* and *N. caninum* infections of BKI compounds in pregnant sheep.

In the **first specific objective**, the *in vitro* phenotype, the virulence in mice and the congenital infection in mice and in sheep of a newly obtained *T. gondii* type II isolate (TgShSp1) were compared

with that of the laboratory type II reference isolate (TgME49). A higher growth rate and formation of cysts *in vitro* and higher pup mortality and vertical transmission after infection of pregnant mice with the laboratory TgME49 isolate compared to the recently obtained TgShSp1 isolate as well as a LD_{50} of 10^3 tachyzoites in mice for the TgME49 isolate suggest an enhanced virulence for the TgME49 isolate probably due to successive passage in cell culture and mice. Therefore, it seems logical to speculate that results from studies using laboratory isolates should be validated with low passage isolates before they can be extrapolated as general features of the respective lineage. The presence of slight clinical signs, but no mortality, in non-pregnant and pregnant mice inoculated intraperitoneally with tachyzoites (1-10^5 tachyzoites) or orally with oocysts (25-2000 oocysts) of the type II isolate TgShSp1 suggest very low virulence in adult mice for this isolate.

In sheep orally infected with oocysts, no differences between these two isolates were found with respect to perinatal mortality or lesions and number of *T. gondii*-positive lambs. The congenital infection of TgShSp1 isolate, measured as perinatal mortality and vertical transmission, was different depending on the challenged host. In mice, mortality in 50% of the pups was observed when a dam was challenged with a high oocyst dose (500 TgShSp1 oocysts), whereas in sheep infected with the same dose of oocysts, mortality occurred in all fetuses. Likewise, mortality of 9% and 27% of the pups was observed in mice after infection with 100 and 25 TgShSp1 oocysts, respectively, while in sheep, infection with 50 and 10 TgShSp1 oocysts triggered mortality in 68% and 66% of the fetuses/lambs. Differences in vertical transmission in the surviving offspring between mice and sheep were only found with the lower oocyst doses (100% after infection with 10 TgShSp1 oocysts in sheep and only 37% in mice after infection with 25 TgShSp1 oocysts). In conclusion, virulence of *T. gondii* type II isolates in mice may not be a good indicator to predict the outcome of experimental infection in pregnant sheep.

In the **second specific objective**, the clinical outcome, immune responses, parasite detection and burden, and lesion severity in placental tissues and foetal brains were investigated in sheep at mid-pregnancy inoculated intravenously with 10^5, 10^4, 10^3, or 10^2 tachyzoites or subcutaneously with 10^4 tachyzoites of the virulent Nc-Spain7 isolate. Pregnant ewes inoculated intravenously with 10^5 tachyzoites showed 100% abortion, with an earlier increase on rectal temperature and IFNγ levels and higher humoral immune responses and parasite load in the foetal brain in these pregnant ewes compared to those challenged intravenously with lower doses. Even intravenous infection with the lowest dose (10^2 tachyzoites) resulted in foetal mortality of 50% of infected ewes and 100% vertical transmission, with no differences on parasite load in the foetal brains compared to infection with 10^5 tachyzoites. On the other hand, pregnant ewes challenged subcutaneously with 10^4 tachyzoites exhibited lower rectal temperature increase and IFNγ levels compared to pregnant ewes challenged intravenously with the same dose of tachyzoites. However, no differences between both routes of administration were found on humoral immune responses, fetal mortality and vertical transmission. These results suggest that a dose beyond 10^5 Nc-Spain7 tachyzoites intravenously implies excessively aggressive conditions for sheep, which might lead to a dismissal of potential active formulations. This dose, 10 times lower than previously assayed, and infection route seems to be sufficient to trigger abortion in all foetuses. Considering these results, we propose the usage of a model based on a maximum dose of 10^5 tachyzoites intravenously in further assays, so that more accurate and realistic conclusions could be obtained in such studies.

In the **third specific objective**, pregnant ewes were orally dosed with BKI-1294 (5 doses of 100 mg/kg every other day) or subcutaneously dosed with BKI-1553 (with two dosages: 1st dose of 35 mg/kg and a week later and a 2nd dose at 10 mg/kg or 7 doses at 10 mg/kg every other day). The efficacy of these drugs administered 48 hours after infection was evaluated in pregnant ewes infected orally with TgShSp1 oocysts (BKI-1294) or intravenously with Nc-Spain7 tachyzoites (BKI-1553).

In addition, drug levels in ewes and foetuses (only BKI-1553) were determined. Subcutaneous administration of BKI-1553 and oral administration of BKI-1294 in pregnant sheep resulted in therapeutic plasma exposures, with maximum concentrations of 11 μM and 2 μM and trough concentrations of 4 μM and 0.4 μM for BKI-1553 and BKI-1294, respectively. In addition, BKI-1553 was able to cross the placental barrier since therapeutic plasma concentrations of 1 μM were found in the foetuses. However, BKI-1553 and more markedly BKI-1294 exhibit a rapid clearance from the plasma. BKI-1294 and BKI- 1553 seems to be safe since no alterations on rectal temperatures, hematological and biochemical parameters, pregnancy or local damage were associated with these drugs. However, differences on safety between both routes of administration were found. While oral administration did not increase rectal temperature nor modified fecal consistency, subcutaneous administration induced the formation of dermal nodules with associated increase on rectal temperature and monocytosis.

The administration of BKI-1294 to *T. gondii* infected ewes and BKI-1553 to *N. caninum* infected ewes resulted in a lower rectal temperature increase, therefore in a lower parasite replication. *In vitro* observations demonstrated the formation of intracellular multinucleated complexes induced by BKI-1294 and BKI-1553 in *T. gondii* and *N. caninum* infected cultures, with increased expression of the tachyzoite antigen SAG1 and the bradyzoite marker BAG1. These multinucleated complexes, composed of multiple pre-zoites unable to separate and form tachyzoites, could influence the cellular and humoral immune responses against *T. gondii* and *N. caninum* since administration of these drugs triggered an increase on IFNγ production and the presence of anti-*T. gondii* SAG1 antibodies. Concerning protection against abortion, in pregnant sheep models of toxoplasmosis and neosporosis in which all untreated ewes aborted, treatment with BKI-1294 of *T. gondii* infected ewes protected in a 76% against perinatal mortality and treatment with BKI-1553 of *N. caninum* infected ewes confered a 37-50% of protection against abortion. As for vertical transmission, while BKI-1553 did not prevent vertical transmission of *N. caninum*, although partially alleviated the effects of infection, by reducing lesions, parasite presence and parasite loads in foetal brains, the BKI-1294 was able to prevent vertical transmission of *T. gondii* in 53% of the lambs.

To summarise, in this Doctoral Thesis a deepeing in the knowledge of the comparative virulence of *T. gondii* type II isolates between pregnant mice and pregnant sheep, the standardization of a pregnant model sheep of neosporosis and the evaluation of the safety and effectiveness of BKI compounds against *T. gondii* and *N. caninum* infection in pregnant sheep have been performed.

CAPÍTULO II

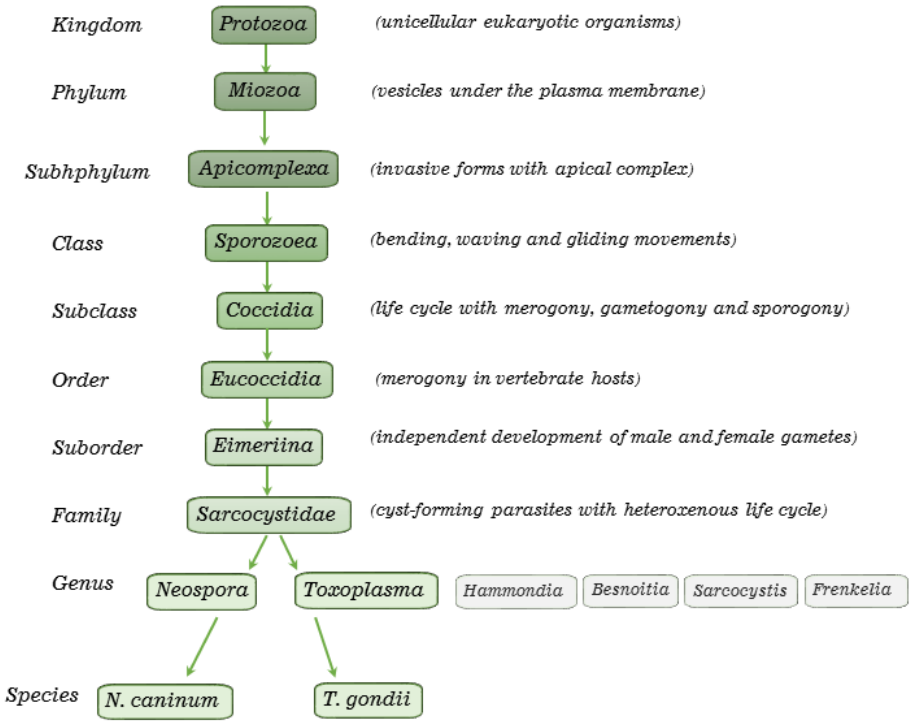
ANTECEDENTES/BACKGROUND

1. Etiological agents

1.1. Taxonomy

Toxoplasma gondii and *Neospora caninum* are obligate intracelular protozoan parasites belonging to subphylum Apicomplexa (Adl *et al.*, 2012). This subphylum is characterized by typical placement of some of its organelles in the anterior part of the cell, forming what is called apical complex. In addition, it is included within Sarcocystidae family, which comprises other genus of cyst-forming apicomplexan parasites with heteroxenous life cycle (*Hammondia*, *Besnoitia*, *Sarcocystis* and *Frenkelia*). Differences on protein, antigenic, structural, molecular and, particularly, biological features between *Toxoplasma*, *Neospora* and the other members of Sarcocystidae family are significant enough to be considered different genus (Ellis *et al.*, 1994; Dubey *et al.*, 2002). The taxonomic classification of *T. gondii* and *N. caninum* and their main characteristics are summarized in Figure 1.

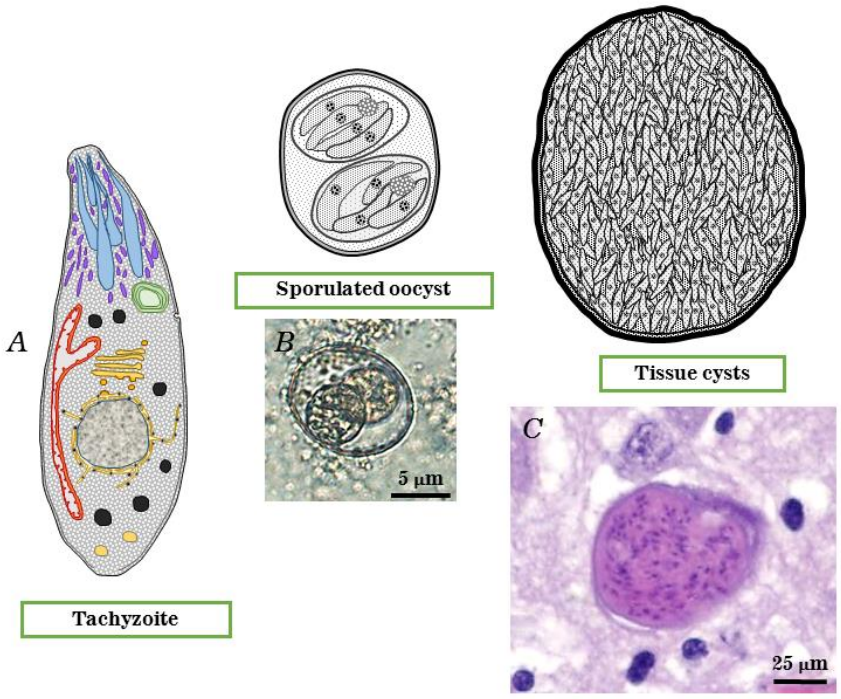
Figure 1 – Taxonomic classification of *Toxoplasma gondii* and *Neospora caninum*.



1.2. Parasite stages

In the *T. gondii* and *N. caninum* life cycle, three different parasite stages have been described: tachyzoites, bradyzoites within tissue cysts and sporozoites within oocysts (Figure 2). Tachyzoites and bradyzoites are the infectious stages that can be found in several tissues from intermediate host, whereas oocysts are shed to the environment in the faeces of the definitive host (cat and other felids in the case of *T. gondii* and dog and other canids in the case of *N. caninum*).

Figure 2 – Graphical representation and microscopic images of *T. gondii* and *N. caninum* parasite stages. Graphical representation of tachyzoite ultrastructure (A). Graphical representation and microscopic image of sporulated oocyst (B). Graphical representation and microscopic image of a brain tissue cyst stained with hematoxylin-eosin (HE) (C).



Tachyzoites are the invasive forms, with high replication rate, being responsible of acute phase of the infection, intraorganic dissemination and tissue damage in the intermediate host (Dubey *et al.*, 1998; Dubey *et al.*, 2006). Tachyzoites shows an ovoid, lunar or ovate morphology depending on the replication stage they are and its size range from 3-7 µm length and 1-5 µm width (Dubey *et al.*, 2002; Dubey, 2010). Ultrastructurally, the tachyzoite has, in addition to typical organelles of any eukaryotic cell, the typical organelles in apicomplexan parasites (Figure 2). The typical organelles in apicomplexan parasites are: the rhoptries and the micronemes distributed in the apical complex, and the dense granules and amylopectin granules homogenously distributed in the tachyzoite (Speer *et al.*, 1999; Dubey, 2010). The rhoptries play an important role on the formation of the parasitophorous vacuole in the host cell. Micronemes are small vesicles which secrete proteins involved in recognition and adhesion to the host cell (Soldati *et al.*, 2001; Hemphill *et al.*, 2013). Dense granules contribute, through secretion products, in formation and maintenance of the parasitophorous vacuole in the host cell (Cesbron-Delauw, 1994; Hemphill *et al.*, 1999).

Bradyzoites are the slow-replicating form of *T. gondii* and *N. caninum* and it is associated to the chronic stage of the infection in the intermediate host. The bradyzoites are approximately 5-8.5 µm x 1-3 µm in size and have the same organelles than the tachyzoites, although there are certain differences in the arrangement and number of secretory organelles (Speer and Dubey, 1989; Dubey *et al.*, 2004; Dubey, 2010). This stage is localized within tissue cysts and formed in the intracytoplasmatic space, mainly in the central nervous system (CNS) and skeletal muscle (Dubey and Lindsay, 1996; Peters *et al.*, 2001; Dubey, 2003; Dubey, 2010). Tissue cysts have round or oval shape with approximately 100 µm diameter and can hold 200 bradyzoites (Figure 2). The cyst wall is thicker and more irregular for *N. caninum* than for *T. gondii* (Speer *et al.*, 1999).

Finally, the sporozoites are localized within the sporulated oocysts, which are the resistant stage of *T. gondii* and *N. caninum*, and shed in faeces of the definitive host (Figure 2). The oocysts are shed

unsporulated to the environment and must undergo a sporogony to be infective. Sporulation occurs within 1 to 5 days depending upon aeration and temperature. Sporulated oocysts from *T. gondii* and *N. caninum*, as well as *Hammondia* spp. are morphologically very similar, with spherical shape and approximately 12 μm length and 11 μm witdh in size. Each sporulated oocyst contain two ellipsoidal sporocysts. Each sporocyst contains four sporozoites and a residual body (Dubey *et al.*, 1998; Dubey *et al.*, 2002).

2. Ovine toxoplasmosis

T. gondii is the causative agent of toxoplasmosis, considered one of the main infectious cause of abortion in sheep (Dubey, 2009b). Likewise, *T. gondii* is a zoonotic parasite with importance in public health (Tenter *et al.*, 2000).

2.1. *T. gondii*: host range and life cycle

T. gondii is a ubiquitous parasite that occurs in most areas of the world. It is capable of infecting an unusually wide range of hosts and many different host cells (Dubey, 2010). The life cycle of *T. gondii* is facultatively heteroxenous (Figure 3). Intermediate hosts are probably all warm-blooded animals including most livestock, and humans. Definitive hosts are members of the family Felidae, for example domestic cats (Dubey and Beattie, 1988).

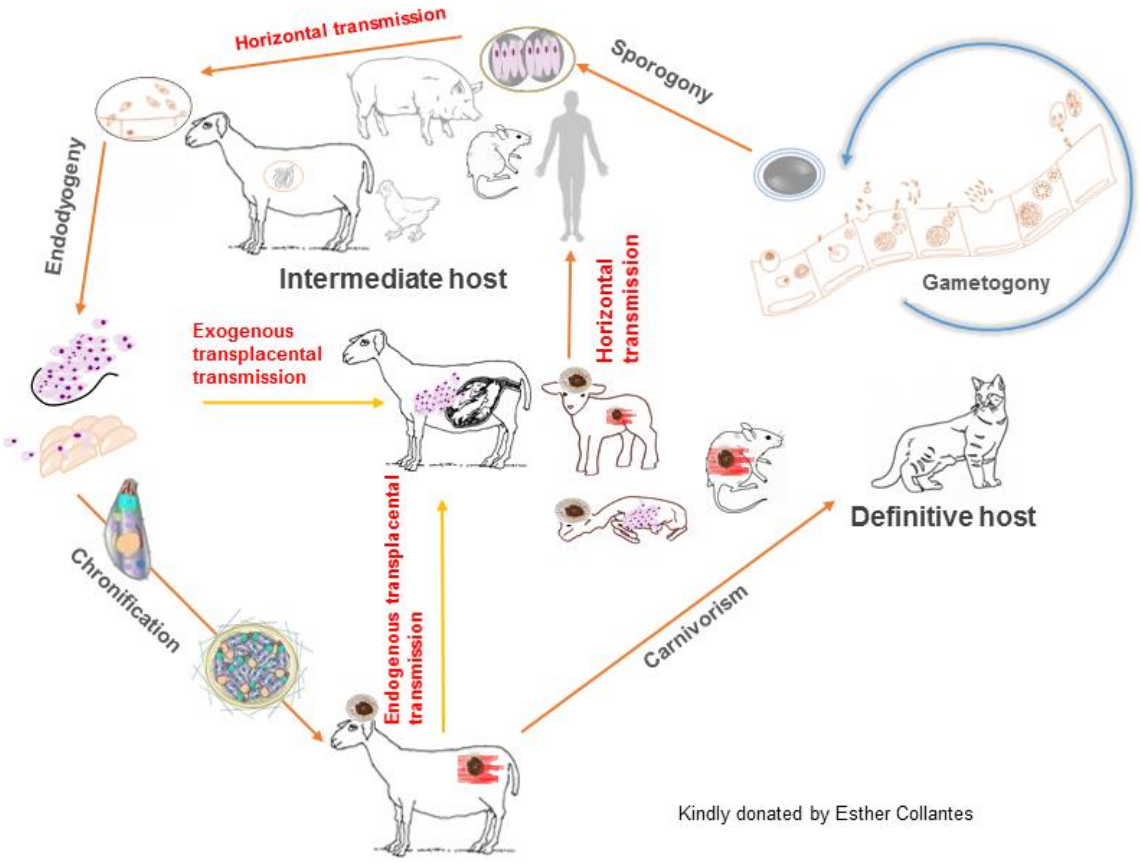
In intermediate hosts, *T. gondii* undergoes two phases of asexual development. In the first phase, tachyzoites multiply rapidly by repeated endodyogeny in many different types of host cells. Tachyzoites of the last generation initiate the second phase of development which results in the formation of tissue cysts. Within the tissue cyst, bradyzoites multiply slowly by endodyogeny (Dubey, 1998). They are located predominantly in the CNS, the eye as well as skeletal and cardiac muscles. However, to a lesser extent they may also be found in visceral organs, such as lungs, liver, and kidneys (Dubey, 1993). Tissue cysts are the terminal life-cycle stage in the intermediate host and are immediately infectious. If ingested by a definitive host, the bradyzoites initiate another asexual phase of proliferation which consists of initial multiplication by endodyogeny followed by repeated endopolygeny in epithelial cells of the small intestine. The terminal stages of this asexual multiplication initiate the sexual phase of the life cycle. Gametogony and oocyst formation also take place in the epithelium of the small intestine. Unsporulated oocysts are released into the intestinal lumen and passed into the environment with the faeces. Sporogony occurs outside the host and leads to the development of infectious oocysts which contain two sporocysts, each containing four sporozoites (Evans, 1992).

There are three infectious stages in the life cycle of *T. gondii*, i.e. tachyzoites, bradyzoites contained in tissue cysts, and sporozoites contained in sporulated oocysts. All three stages are infectious for both intermediate and definitive hosts which may acquire a *T. gondii* infection mainly via one of the following routes: (A) horizontally by oral ingestion of infectious oocysts from the environment, (B) horizontally by oral ingestion of tissue cysts contained in raw or undercooked meat or viscera of intermediate hosts, or (C) vertically by transplacental transmission of tachyzoites (Dubey, 1998) (Figure 3).

Thus, *T. gondii* may be transmitted from definitive to intermediate hosts, from intermediate to definitive hosts, as well as between definitive and between intermediate hosts (Figure 3). The prevalence of *T. gondii* infections is not confined to the presence of a certain host species. Its life cycle may continue indefinitely by transmission of tissue cysts between intermediate hosts (even in

the absence of definitive hosts) and also by transmission of oocysts between definitive hosts (even in the absence of intermediate hosts).

Figure 3 – Life cycle and routes of transmission of *T. gondii*



2.2. Parasite isolates and intraspecific variability

Many *T. gondii* isolates have been obtained from different host species around the world, including humans, as well as from different organs and parasite stages (Dubey, 2010). *T. gondii* presents three different clonal lineages classified as I (such as RH and GT1 isolates), II (such as TgME49 and PRU isolates) and III (such as VEG isolate). While clonality is the predominant pattern in North American and European isolates, South American isolates are highly diverse and some of them divergent from the three major lineages, referred to as 'exotic or atypical genotypes' (Shwab *et al.*, 2014).

A variety of different methods exist to genotype *T. gondii* isolates, such as multilocus enzyme electrophoresis (Dardé *et al.*, 1992), microsatellite markers (Ajzenberg *et al.*, 2002) and restriction fragment length polymorphism (RFLP) analysis (Su *et al.*, 2006), and they have distinct advantages and disadvantages. All of the above methods underestimate the true rate of polymorphism and hence may misclassify variants owing to homoplasy or insufficient resolving power. In contrast, direct sequencing of genomic regions reveals the complete genetic diversity including single nucleotide polymorphisms (SNPs) and small insertions and deletions. The obvious disadvantage of sequence-based typing is its increased cost and need for access to sophisticated technology (Sibley *et al.*, 2009).

Laboratory mice are generally sensitive to *T. gondii* infection and are often used as the preferred animal model to determine the virulence of the parasite (Saraf *et al.*, 2017). Type I are highly virulent in mice (LD_{100} of 1 tachyzoite) whereas types II and III exhibited median lethal doses (LD_{50}) that

range from 10^2 to 10^5 (Saeij *et al.*, 2006). Type II *T. gondii* is the most prevalent in all hosts in Europe, including sheep (Dumètre *et al.*, 2006; Halos *et al.*, 2010b; Su *et al.*, 2010).

2.3. Transmission

Transmission of *T. gondii* in sheep was historically linked to the contamination of feed and water with cat faeces (Plant *et al.*, 1974; Faull *et al.*, 1986; Innes *et al.*, 2009) (Figure 3). There is a widespread environmental contamination with *T. gondii* oocysts (Dabritz *et al.*, 2007), and field investigations have shown an association between cats on farms and the exposure of sheep to *T. gondii* (Skjerve *et al.*, 1998). Likewise, environmental contamination with oocysts also explains serological surveys in which sheep show an increasing seroprevalence to *T. gondii* with age (Waldeland, 1977; Lunden *et al.*, 1994).Therefore, after primoinfection with *T. gondii* during pregnancy, exogenous transplacental transmission is the most frequent mode of transmission (Trees and Williams, 2005).

It is estimated that less than 4% of chronically infected sheep transmit *T. gondii* to offspring (Dubey and Beattie, 1988; Buxton *et al.*, 2006; Buxton *et al.*, 2007). Several studies indicate that endogenous transplacental transmission is rare and plays a minor role in transmission of *T. gondii* in sheep (Watson and Beverley, 1971; Munday, 1972; Rodger *et al.*, 2006). By contrast, some studies have suggested that chronically infected sheep can transmit infection to offspring, but despite a large proportion of abortions and lambs being *T. gondii* positive by polymerase chain reaction (PCR) was found, there is a lack of serological and pathological data (Duncanson *et al.*, 2001; Williams *et al.*, 2005).

2.4. Pathogenesis, clinical signs and lesions

Despite decades of research, there are remain gaps in knowledge about this disease, especially regarding the pathogenesis of abortion in sheep (Benavides *et al.*, 2017). The stage of pregnancy when transplacental transmission of *T. gondii* takes place is important in determining the clinical outcome. If infection occurs early in gestation, when the foetal immune system is relatively immature, foetal death is likely to occur. Infection at mid-gestation can result in birth of a stillborn or weak lamb which may have an accompanying small mummified foetus, whereas infection in later gestation may result in birth of a live, clinically normal, but infected lamb (Watson and Beverley, 1971; Hartley and Moyle, 1974; Blewett *et al.*, 1982).

The reason for this variation in the pathogenesis of abortion is unclear, but several hypotheses have been proposed, all of them related to the immune response of the host. Both maternal and foetal immune responses are subject to change during pregnancy (Entrican, 2002), but the precise role of immunity is not known. While foetal immunocompetence evolves throughout gestation, it is not until mid-gestation onwards that more specific immune responses can be elicited (Castaño *et al.*, 2016). Therefore, from mid-gestation there is more control of parasite replication in foetal tissues and hence greater foetal survival. At the same time the maternal immune response also influences the pathogenesis of any infection. As such, it is widely accepted that the placenta is under immunomodulation during pregnancy and that local and peripheral immune responses differ at this time; however, how immunity varies during gestation and the influence of the immune response on the outcome of *T. gondii* infection remain in large part to be elucidated (Benavides *et al.*, 2017).

The histopathological changes in the placenta and foetal viscera instigated after *T. gondii* infection are basically the same, and comprise of non-purulent inflammation and necrosis, regardless of the

period of gestation when infection occurred. However, the stage of gestation influences the severity of these lesions and the time post infection when they appear (Buxton *et al.*, 1982; Buxton and Finlayson, 1986). In the foetal tissues, the brain is the most frequent location of lesions, which appear as scattered foci of necrosis following infection in early pregnancy, while in infections initiated in older fetuses foci of gliosis with central areas of necrosis and occasional mineralization predominate. Skeletal muscle, heart, lung and liver are the other target locations where lesions may be found. When evaluating lesion severity, those occurring due to infection in mid-gestation are more severe in terms of size and involvement of inflammatory cells. These findings appear to be related directly to the maturation of the foetal immune system, as from mid-gestation the immune system becomes able to respond to the infection and promote a specific inflammatory reaction (Castaño *et al.*, 2016). In the placenta lesions are mainly of necrosis and the number and size of lesions increases as gestation advances. Infection in early gestation causes necrotic lesions affecting the caruncular septa and there is an increase in the number of inflammatory cells, mainly macrophages and lymphocytes, in the foetal villi adjacent to the lesions. Infection later in gestation is associated with greater damage to the placenta, which shows more frequent and more extensive necrotic foci involving both maternal and foetal tissues. There is also a greater inflammatory component, which is mainly mononuclear and more evident in the foetal villi, but also includes scant neutrophils in maternal tissue. These lesions are usually appreciable grossly as multiple white foci in the cotyledons (Buxton, 1998). Inflammation in the maternal compartment of the placenta is largely similar during the different stages of gestation, and is not the main pathological feature. In the foetal part of the placenta, the infiltration of inflammatory cells is greater in late gestation than in midgestation and very scant in early pregnancy, as is the case in the foetal viscera (Castaño *et al.*, 2016; Benavides *et al.*, 2017).

Maternal and foetal immune responses to the infection are key players in its outcome, and the influence of stage of gestation is also crucial. The maturation of the foetal immune system from midgestation contributes to control of multiplication of the parasite in the foetal viscera and explains the increased number of inflammatory cells as the main component of the lesions at this stage of pregnancy. While this influence on the pathogenesis of toxoplasmosis is generally accepted, the role and modulation of the maternal immune response during gestation is a more controversial issue, not only in toxoplasmosis, but in other infections occurring during pregnancy. The T helper (Th)1/Th2 paradigm of immunoregulation by $CD4^+$ Th lymphocytes, which has been used to explain a shift towards Th2 immunity in the maternal immune response from mid-pregnancy, has been under reappraisal in recent years, as it fails to explain several immunological aspects of gestation, such as the role of innate immunity or the role of Th1- derived cytokines and/or cells in supporting gestation. The combination of these variations in the foetal and maternal immune response may explain why vertical transmission is less common in early pregnancy and why it takes longer for the parasite to reach the fetus, as the peripheral maternal immune response may control dissemination of the organism, but once it invades the fetus, with an immature immune response, it invariably causes its death. In contrast, in late gestation, the parasite disseminates more easily to the placenta, possibly facilitated by the modulation of the maternal immune response, and therefore, invades the fetus earlier. Probably, also due to local immunomodulation, the organism can multiply in the placenta more easily than in earlier stages of gestation, and so cause more severe lesions. However, once it invades the foetus, its dissemination is controlled by the more mature foetal immune response, which can mobilize an adequate contingent of inflammatory cells. The specific mechanisms, which are modulated in the maternal immune response, innate or adaptive, peripheral or local, as well as those responsible for the parasite control in the fetus, remain to be elucidated (Benavides *et al.*, 2017).

Recent experimental studies have defined an unacknowledged clinical presentation of ovine toxoplasmosis, where abortions occur during the acute phase of infection, resulting in as high as 100% foetal loss in susceptible sheep (Trees *et al.*, 1989; Owen *et al.*, 1998a; Castaño *et al.*, 2014; Castaño *et al.*, 2016). The pathogenesis of this clinical form differs from that of classically described ovine toxoplasmosis. It can occur in any period of gestation and is characterized by the occurrence of abortion 7-12 days after infection (Castaño *et al.*, 2016), while "classical" abortion due to the parasite is likely to occur from the 28th day (Buxton, 1998). This interesting clinical presentation has been noted only following experimental infection; however, it is thought likely to cause a significant proportion of abortions caused by *T. gondii* under natural conditions. The differences between these two presentations of abortion are also evident at the pathological level. While necrotic lesions with a variable degree of inflammatory infiltration are the hallmark histological finding in the placenta and foetal brain in "classical" *T. gondii* abortions, in abortions during the acute phase of the disease, placental infarcts associated with thrombosis in the maternal vessels and periventricular leukomalacia in foetal brain are found. Although the pathogenesis of this clinical form is still largely unknown, it seems reasonable to hypothesize that the vascular lesion in the placenta contributes to foetal hypoxia (Castaño *et al.*, 2014). The fact that these abortions occur shortly after infection, suggesting that there is not enough time for *T. gondii* to invade and multiply in the placenta, would seem to be relevant. While it is probable that the parasite plays a direct role in the pathogenesis of this clinical form, it is also tempting to suggest that there are other factors, such as the maternal immune response, both local and peripheral, involved in its pathogenesis. The cause of the thrombosis, as related to *T. gondii* infection, needs to be investigated (Benavides *et al.*, 2017).

2.5. Immunity

During pregnancy the ewe´s immune responses are modulated to accommodate the presence of the semiallogeneic fetus and as a result maternal immunity at the materno-fetal interface suppresses mechanisms that activate inflammatory cells (Entrican and Wheelhouse, 2006). Thus, there is minimal maternal expression of cytokines, such as interleukin 2 (IL-2), tumour necrosis factor alpha (TNFα) and gamma interferon (IFN-γ) (Entrican and Wheelhouse, 2006). However, while permitting a successful pregnancy these mechanisms also represent a vulnerability, making the placenta and foetus peculiarly susceptible to certain pathogens. Thus, when *T. gondii* circulate in the blood of a pregnant ewe they are able to become established in the placenta where they parasitise the maternal caruncular septa in the placentome before invading the adjacent trophoblast cells of the fetal villi, from where they can spread to the rest of the foetus (Buxton and Finlayson, 1986).

Following initial infection of the ewe both the innate and adaptive immune responses work together to limit multiplication of the fast replicating tachyzoite stage (Innes and Vermeulen, 2006). *T. gondii* are able to stimulate innate immune mechanisms directly upon entry into the host. This direct stimulation of macrophages results in production of interleukin 12 (IL-12) which directly stimulates natural killer (NK) cells to produce IFN-γ (Gazzinelli *et al.*, 1993). IFN-γ is known to be important in inhibiting the intracellular multiplication of *T. gondii* and in addition will create the appropriate cytokine microenvironment for the priming of the adaptive immune response towards a Th-1 type pro-inflammatory immune response (Innes and Vermeulen, 2006). Studies in sheep using the technique of chronic lymphatic cannulation allowed the study of the development of a primary immune response to *T. gondii* in real time (Innes and Wastling, 1995).

Interestingly, the first immune response detected within 48 hours of *T. gondii* inoculation was IFN-γ (Innes *et al.*, 1995). Four to five days after inoculation of *T. gondii*, T cells responding to the

infection were detected in the efferent lymph. Using phenotypic markers specific for ovine lymphocytes the experiment showed that initially the predominant lymphocyte population comprised CD4+ T cells (Innes *et al.*, 1995). At day 9–10 postinoculation, the lymphocyte population peaked (Buxton *et al.*, 1994). At peak lymphocyte output the predominant population switched to CD8+T cells and *in vitro* studies showed that these activated CD8+ T cells were able to inhibit multiplication of *T. gondii* infected autologous ovine target cells directly (Innes *et al.*, 1995). Following the peak lymphocyte response, the parasite was no longer detected in the efferent lymph indicating that the immune system of the sheep had successfully controlled the infection (Innes and Wastling, 1995). Specific antibodies to *T. gondii* were detected from day 10-12 after inoculation indicating that cell mediated immune responses involving, CD4+, CD8+ T cells and IFN-γ are important in protective immunity and recovery from a primary infection and specific antibody may play more of a role in protection against a secondary infection (Innes and Vermeulen, 2006). These results, taken together with the work published by Watson and Beverley (1971), showing that ewes infected in one pregnancy are unlikely to have infected lambs in subsequent pregnancies.

2.6. Prevalence and economic impact

Antibodies to *T. gondii* have been found in sheep worldwide (Dubey, 2009b). However, the data are not comparable among different locations because of the use of different serological tests and different cut-off values used to determine seropositivity. The prevalence of antibodies in ewes was more than twice that in lambs, but results were dependent on the age of the sampled lambs (Lunden *et al.*, 1994; Gorman *et al.*, 1999; Figliuolo *et al.*, 2004; Rozette *et al.*, 2005; Dumètre *et al.*, 2006; Ragozo *et al.*, 2008). Seroprevalence was shown to increase with age, reaching 95% in 6-year-old ewes in some flocks (Dubey and Kirkbride, 1989), suggesting that most animals acquire infection post-natally. In general, most sheep acquired infection before 4 years of age, but one-third of old ewes were still seronegative in highly endemic flocks (Dubey and Kirkbride, 1989). Prevalence was also higher in ewes on farms where epizootics of abortions were reported (Dubey and Kirkbride, 1989). Seroprevalence in intensively managed sheep was lower than in semi-intensive management (Savio and Nieto, 1995; Romanelli *et al.*, 2007; Samraa *et al.*, 2007; Ragozo *et al.*, 2008).

In Spain, the individual seroprevalence rate of *T. gondii* infection in sheep ranged from 11% to 57% (Mainar *et al.*, 1996; Marca *et al.*, 1996; Panadero *et al.*, 2010; Garcia-Bocanegra *et al.*, 2013; Díaz *et al.*, 2014; Almería *et al.*, 2018) and the flock seroprevalence rate is estimated on 84% (García-Bocanegra et al., 2013). Regarding abortions, between 5.4% and 23% of ovine abortions in Spain were associated with *T. gondii* (Pereira-Bueno *et al.*, 2004; Moreno *et al.*, 2012). Actual losses in lambs due to toxoplasmosis are difficult to estimate because (1) the disease is usually sporadic, (2) only a small number of aborted lambs are submitted for diagnosis, (3) those submitted may be inadequately examined, (4) unsuitable material may be sent for diagnosis, (5) the serologic test may not be specific, and (6) toxoplasmosis does not produce clinical disease in the ewe, so this disease does not alarm the farmer as much as other bacterial and viral infections. In the EU, losses due toxoplasmosis was estimated around 1.5 millions of lambs per year, representing a significant loss to producers (Innes *et al.*, 2009).

2.7. Diagnosis

Different serological techniques have been used for the detection of *T. gondii* infection in sheep (Dubey, 2009b). An excellent correlation between modified agglutination test (MAT) and indirect

immunofluorescent test (IFAT) has been found (Marca *et al.*, 1996). In addition, various enzyme-linked immunosorbent assay (ELISA) methods using crude, fractionated, or recombinant antigens have been used to detect *T. gondii* antibodies in ovine sera. Tenter *et al.* (1992) compared ELISA based on crude and recombinant antigens and found varying degrees of specificity in naturally and experimentally infected sheep. Caballero-Ortega *et al.* (2008) reported a good correlation between their in-house crude *T. gondii* antigen ELISA and Western blot (WB). They also reported on the value of an avidity ELISA to investigate the duration of infection in sheep.

For the diagnosis of toxoplasmosis in ovine abortions, detection of *T. gondii* antibodies in foetal fluids or serum is useful (Arthur and Blewett, 1988). In addition, foetal tissues should be submitted for molecular detection of *T. gondii* deoxyribonucleic acid (DNA) by PCR (Hurtado *et al.*, 2001). Although foetal serology and DNA detection are useful aids, histopathology is essential to establish a cause – effect association, because *T. gondii* can be passively transmitted transplacentally and fetuses can die due to other causes (Dubey, 2009b).

The diagnosis of ovine abortions during the acute phase of the disease (7-11 days after infection) is actually problematic since specific antibodies against *T. gondii* in the maternal sera have not been detected, and the parasite is not in placental and foetal tissues or the parasite burden is very low, which considerably hinders the diagnosis of the disease (Castaño *et al.*, 2016). Therefore, the histological evaluation of the placental tissues looking for placental infarcts is essential (Castaño *et al.*, 2014).

3. Bovine neosporosis

N. caninum is the causative agent of bovine neosporosis, considered one of the main infectious agent of abortion in cattle, causing important economic losses in cattle industry worldwide (Dubey *et al.*, 2017).

3.1. *N. caninum*: host range and life cycle

N. caninum has a facultative heteroxenous life cycle with two different hosts, a definitive host and an intermediate host, in which sexual and asexual multiplication are found, respectively. To date, as definitive hosts are been described, the domestic dog (McAllister *et al.*, 1998), the coyote (Gondim *et al.*, 2004), the dingo (King *et al.*, 2010) and the wolf (Dubey and Schares, 2011). On the other hand, as intermediate hosts are involved, mainly, cattle and sheep (Thilsted and Dubey, 1989), being the former the main intermediate host. Nevertheless, recent evidence suggests that *N. caninum* is also an important abortifacient in sheep (Moreno *et al.*, 2012; Benavides *et al.*, 2014; González-Warleta *et al.*, 2014). However, although *N. caninum* infection has been detected by different diagnostic thecniques in a wide range of wild and domestic animals, isolation has been only possible in a few host species (cattle, European Bison, sheep, white-tail deer, horses and dogs) (Dubey *et al.*, 2007; Dubey and Schares, 2011). Conversely, although *N. caninum* infection was experimentally reproduced in macaques, there is no evidence that *N. caninum* could infect humans (Dubey *et al.*, 2007; McCann *et al.*, 2008).

Oocyst shedding by dogs, the most important definitive host in the domestic cycle, has been described by several authors (McAllister *et al.*, 1998; Dubey *et al.*, 2007; Dubey and Schares, 2011). Once in the environment, oocysts must undergo sporulation to be infective. Similarly to other coccidia, oocysts have a very high environmental resistance (Uzeda *et al.*, 2007; Alves Neto *et al.*,

2011). The schizogony of *N. caninum* takes place in the intermediate host. Following ingestion of food or water contaminated with sporulated oocysts, sporozoites are released in the gut and transformed into tachyzoites. Later, they reach bloodstream, through mesenteric lymph nodes, taking place the intraorganic dissemination of the parasite. Tachyzoites can invade a wide range of tissues and organs, such as heart, lungs, liver, skeletal muscle, placenta, brain or skin (Dubey and Lindsay, 1996; Maley *et al.*, 2003; Dubey *et al.*, 2006). Over time, the host build an immune response capable of eliminate the majority of the tachyzoites, although a few of them evade immune response through conversion to latent bradyzoite stage, mainly in the CNS and skeletal muscle, thus starting the chronic phase of the infection (Peters *et al.*, 2001; Buxton *et al.*, 2002). In this way, *N. caninum* persists in the host for long periods of time without showing clinical signs (Peters *et al.*, 2001; Buxton *et al.*, 2002).

The ingestion of infected tissues containing bradyzoites is the only mode of transmission of infection between the intermediate host and the definitive host, thus closing the life cycle (Dubey and Schares, 2011). In addition, *N. caninum* can also be transplacentally transmitted in the intermediate host (Dubey, 2003). Finally, the presence of a sylvatic life cyle in addition to the already known domestic life cycle has been described (Gondim, 2006). *N. caninum* can circulate between both cycles, being possible the transmission of *N. caninum* from wild animals to the dogs and after that, to cattle, as well as from cattle to wild canids and from this to the wild ruminants (Gondim *et al.*, 2004d).

3.2. Parasite isolates and intraspecific variability

Since the first *N. caninum* isolation in 1988 (Nc-1 isolate) (Dubey *et al.*, 1988), the total number of isolates obtained has been relatively low (around 100). So far, *N. caninum* isolates have been obtained from domestic (cattle, sheep, dog, horses) and wild (deer, buffalo, bison and wolf) animals (Dubey and Schares, 2011; Dubey *et al.*, 2014). In Spain, SALUVET research group has obtained a large number of isolates (10), mainly from congenitally infected but healthy calves (Regidor-Cerrillo *et al.*, 2008; Rojo-Montejo *et al.*, 2009).

Several authors have demonstrated differences after *in vitro* assays on invasion and proliferation rate (Schock *et al.*, 2001; Regidor-Cerrillo *et al.*, 2011; Dellarupe *et al.*, 2014b), as well as in dissemination and transmigration ability (Collantes-Fernández *et al.*, 2012). Similarly, studies in experimental mice models revealed differences on virulence between isolates by evaluating parasite load, morbidity, vertical transmission and neonatal mortality (Lindsay *et al.*, 1995; Atkinson *et al.*, 1999; Collantes-Fernández *et al.*, 2006a; Rojo-Montejo *et al.*, 2009; Regidor-Cerrillo *et al.*, 2010; Dellarupe *et al.*, 2014a). In addition, there is a large correlation between invasion and proliferation rate and the pathogenesis in mice (Regidor-Cerrillo *et al.*, 2011; Dellarupe *et al.*, 2014b) and even, between observations in mice and bovine models, at least with Nc-1, Nc-Spain1H and Nc-Spain7 isolates. Altogether, these studies allow to classify some isolates according to their virulence. Thereby, it has been confirmed a high virulence in Nc-Liverpool and Nc-Spain7 isolates and a low virulence in Nc-Spain1H isolate (Rojo-Montejo *et al.*, 2009; Regidor-Cerrillo *et al.*, 2010; Regidor-Cerrillo *et al.*, 2011; Dellarupe *et al.*, 2014a; Dellarupe *et al.*, 2014a; Dellarupe *et al.*, 2014b).

The classification of *N. caninum* isolates based on the virulence, opens new research fields, since their comparisons allow to identify new potential virulence factors, whose understanding is essential for the development of pharmacological and immunoprophylatic tools capable of blocking essential processes for the parasite.

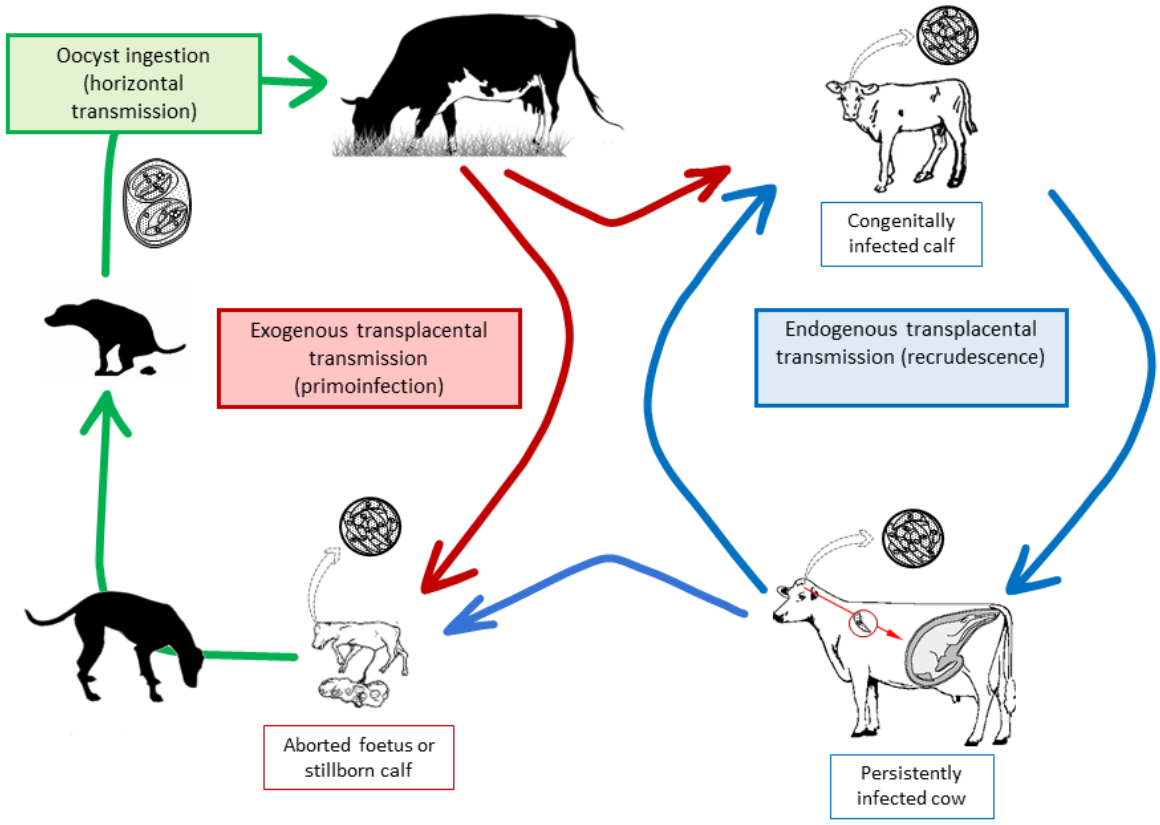
3.3. Transmission

Two different modes of *N. caninum* transmission are described in cattle: horizontal and vertical. Horizontal transmission, also called postnantal, takes place through ingestion of food or water contaminated with oocysts shed by the definitive host. In turn, vertical or transplacental transmission occurs when in a pregnant female, tachyzoites cross the placental barrier and reach the foetus (Figure 4). Transplacental transmission (congenital transmission) is the most efficient mode of transmission, since percentages of transmission of 40-95% have been described (Dubey *et al.*, 2007), playing a key role in the propagation and maintenance of the disease in a flock. In addition, a congenitally infected cow can transmit the infection to the offspring, repeatedly, in different pregnancies (Boulton *et al.*, 1995; Wouda *et al.*, 1998; Guy *et al.*, 2001; Fioretti *et al.*, 2003).

From the point of view of the origin of the transplacental infection, two different modes of transmission have been described, exogenous and endogenous (Trees and Williams, 2005). Both modes of transmission exhibit different pathological and epidemiological consequences, and therefore different control measures. Endogenous transplacental transmission takes place after recrudescence of a chronic infection during pregnancy in a persistently infected female. By contrast, exogenous transplacental transmission occurs in cows being infected by ingestion oocysts for the first time during pregnancy and being transmitted to the offspring. Chronically infected herds show an endemic pattern of abortions. By contrast, herds showing a first exposure to sporulated oocyst infection show an abortion outbreak (30-57%), due to an exogenous transplacental transmission (Trees and Williams, 2005; Dubey *et al.*, 2007). Consequently, endogenous transplacental transmission is the most frequent mode of transmission in many herds, while controversy exists about the significance of exogenous transplacental transmission on the establishment of a chronic infection (McCann *et al.*, 2007; Dijkstra *et al.*, 2008).

Notwithstanding the above, vertical transmission rates decline with age. This, together with the fact that transplacental transmission does not happen in 100% of the cases, means that without oocyst as source of infection (horizontal transmission), the disease tends to disappear over generations (Dubey *et al.*, 2007; Eiras *et al.*, 2011). Thus, exogenous and endogenous modes of transmission seem to complement each other to avoid this phenomenon. In fact, different epidemiological scenarios have been described in Spain, with exogenous and endogenous modes of transmission being present independently or in combination (Rojo-Montejo *et al.*, 2009).

Figure 4 – Graphical representation of *N. caninum* routes of transmission



3.4. Pathogenesis, clinical signs and lesions

N. caninum infection in non-pregnant cattle is usually asymptomatic, while in pregnant animals the most relevant clinical sign is the abortion (Dubey, 2005). *N. caninum* is a primary pathogen in terms of triggering the abortion, although mechanism by which the foetus dies and triggers the abortion is not completely understood. During parasitaemia, caused by primoinfection or most commonly by reactivation of a chronic infection during pregnancy, tachyzoites are disseminated, invading different tissues and being able to cross the placental barrier and reach the foetus (Dubey *et al.*, 2006). Different mechanisms causing foetal death have been postulated. On the one hand, the direct action of *N. caninum*, through invasion and replication in placental and foetal tissues, would lead severe lesions, affecting foetal survival. In turn, placental lesions could lead abortion due to reduced oxygen and nutrients supply, either by tachyzoite replication or by maternal inflammatory immune response in response to *N. caninum* infection (Dubey *et al.*, 2006). On the other hand, once *N. caninum* invade placental and foetal tissues, the different outcomes of infection (abortion, birth of calves showing neurological signs and most frequently, birth of clinically healthy but congenitally infected calves) will depend on different factors related to both the parasite and the host, being the most important the period of gestation in which the infection occurs, directly related to the maternal and foetal immunity. Abortion can occur from the third month of pregnancy, albeit it is more frequent between the 5th and the 7th months of pregnancy (Dubey *et al.*, 2007; Almería and López-Gatius, 2013). If infection occurs in the first term of gestation, the foetus is often reabsorbed, and clinically a repeat breeding is observed. However, no association was found between *N. caninum* infection and early reproductive failure (Almería and López-Gatius, 2013). On the other hand, if foetal death takes

place between 3 and 8 months of pregnancy, foetus is usually expelled exhibiting a moderate autolysis. However, foetuses dying before 5th month of pregnancy could exhibit mummification and remain in utero for months. If infection occurs later in gestation, from 5th month of pregnancy, risk of foetal death decrease and the more frequent clinical sign is the delivery of healthy, but congenitally infected, calves, which generate precolostral antibodies (Quintanilla-Gozalo *et al.*, 2000; Williams *et al.*, 2000).

In a limited number of cases, weak infected calves showing neurological signs, ranging from slight ataxia to complete paralysis could be delivered. In the most severe cases, dorsal spine malformations, hycrocephalus or pneumonia can appear (Dubey *et al.*, 1992; Bryan *et al.*, 1994; Gunning *et al.*, 1994). These clinical signs are more likely to appear during the first week after delivery, although it could be delayed until two weeks after delivery (Dubey and Lahunta, 1993; Duivenvoorden and Lusis, 1995) and evolve until total paralysis and death of the animal during first month after birth (Dubey *et al.*, 1992; Gunning *et al.*, 1994; Dubey *et al.*, 2006).

Lesions associated to infection can be observed in different organs, depending on the stage and severity of infection, being, in general, inflammatory non suppurative lesions. In placental tissues, necrotic foci and inflammatory areas with mononuclear cells are often observed, although chronicity could lead to regeneration with connective tissue, hyperplasia, fibrosis and even, calcification of necrotic foci (Barr *et al.*, 1994; Maley *et al.*, 2003). Placental lesions are more severe and necrosis more extensive when foetal death takes place compared to those from ewes that give birth (Gibney *et al.*, 2008).

Regarding lesions from foetuses or congenitally infected calves and lambs, they are mainly found in the CNS. The lesions consist of multifocal lymphocyte infiltration, often surrounding blood vessels (perivascular cuffing), microgliosis and presence of astrocytes, likely with necrosis and mineralization (Barr *et al.*, 1991; Dubey and Lindsay, 1996). This type of non-suppurative inflammation is also observed in heart, liver tissues and less frequently in kidney, skeletal muscle and lung tissues (Dubey *et al.*, 2006). *N. caninum* associated lesions are more severe in aborted foetuses from an epidemic pattern of abortions compared to those from endemic abortions. In congenitally infected calves without clinical signs is also possible to find lesions, but less frequently, less severe and mainly in the CNS (Barr *et al.*, 1991; Bryan *et al.*, 1994; Sawada *et al.*, 2000). Lesions can be associated in most cases to the presence of tachyzoites or cysts in the tissues, mainly in the CNS (Dubey and Lindsay, 1996), and less frequently in the heart, liver and skeletal muscle (Wouda, 1997; Morales *et al.*, 2001; Peters *et al.*, 2001).

3.5. Immunity

N. caninum infection in cattle triggers a combination of innate and adaptative immune responses trying to control parasite dissemination in the animals (Innes *et al.*, 2001b; Almería *et al.*, 2003; Staska *et al.*, 2003; Andrianarivo *et al.*, 2005; Rosbottom *et al.*, 2008; Bartley *et al.*, 2013). These mechanisms are activated early after infection, and components of the innate immunity such as dentritic cells, NK cells and macrophages are mobilized. These cell types act as the first line in defence, destroying infected cells and releasing cytokines such as IL-12 and IFN-γ (Boysen *et al.*, 2006; Klevar *et al.*, 2007; Strohbusch *et al.*, 2009b; Feng *et al.*, 2010; Dion *et al.*, 2011). Parasite recognition by antigen presenting cells and early cytokine production is essential for the control of tachyzoite proliferation early after infection.

Intracellular location of *N. caninum* suggests that cell mediated immune response is the most important component related to protection (Hemphill, 1999; Innes *et al.*, 2000). Thus, control of *N.*

caninum infection is associated with a Th1 immune response, in which different proinflammatory cytokines, mainly IFN-γ, and T cells are involved (Khan *et al.*, 1997; Hemphill *et al.*, 2006). Importance of IFN-γ has been demonstrated *in vitro* and *in vivo*, in which inhibition of *N. caninum* proliferation was found after IFN-γ addition in cell culture (Innes *et al.*, 1995; Yamane *et al.*, 2000), as well as a higher susceptibility to infection in a murine model after treatment with anti-IFN-γ antibodies (Khan *et al.*, 1997; Baszler *et al.*, 1999).

Finally, the role of humoral immune response in the control of neosporosis is unclear and remains to be elucidated. It is possible that antibodies specifically generated against the parasite have several functions, such as opsonisation of extracellular parasites, with the consequent phagocytosis by macrophages, similarly as observed in toxoplasmosis (Sibley *et al.*, 1985).

In fact, knockout -B cell-deficient- mice, showed a higher susceptibility to *N. caninum* infection (Eperon *et al.*, 1999). In any case, antibody levels fluctuate during gestation (Stenlund *et al.*, 1999; Guy *et al.*, 2001; Andrianarivo *et al.*, 2005; Nogareda *et al.*, 2007), which might be associated with the proliferation of the parasite (Weston *et al.*, 2005; Innes, 2007).

3.6. Prevalence and economic impact

At present, there are relatively large number of seroprevalence studies of *N. caninum* in cattle, and variable prevalence rates have been described depending on geographic location and purpose of the breeding (beef cattle *vs* dairy cattle) (Dubey *et al.*, 2007). However, data are difficult to compare since they were obtained using different techniques, and very variable experimental designs and sample sizes. Despite this, in Europe, Nordic countries and Germany have the lowest seroprevalence rates (0.7-12.8%), while in South America, Argentina and Brazil have the highest seroprevalence rates (80.9-97.2%) (Dubey and Schares, 2011).

In Spain, the first studies showed very high herd prevalences, of 55% and 83% in beef cattle and dairy cattle, respectively. Individual prevalence rates showed values of 18% in beef cattle and until 36% in dairy cattle (Mainar-Jaime *et al.*, 1999; Quintanilla-Gozalo *et al.*, 1999). These results were similar to those found in Galician cattle, where herd seroprevalence rate reached 88% in dairy cattle and 77% in beef cattle. Individual seroprevalence ranged from 16 to 22% in dairy cattle herds and was of 25% in beef cattle herds (Gonzalez-Warleta *et al.*, 2008; Eiras *et al.*, 2011).

Regarding aborted bovine foetuses, it is estimated that between 12 and 40% of them are infected with *N. caninum* around the world (Dubey *et al.*, 2007). In Spain, *N. caninum* infection was detected in 58% of analyzed foetuses using different diagnostic techniques (Gonzalez *et al.*, 1999). In a later study, 39% of analyzed foetuses were positive, at least, by one of the diagnostic techniques used. These results revealed the importance of *N. caninum* as an abortifacient agent in our country, even ahead of bovine viral diarrhoea virus (Pereira-Bueno *et al.*, 2003).

Bovine neosporosis, thus, has been regarded one of the main infective causes of reproductive failure worldwide (Dubey and Schares, 2011; Reichel *et al.*, 2014), causing important economic impact in dairy and meat industry. Economic losses are related to abortion, neonatal mortality, as well as other indirect effects such as prolonged calving intervals, decrease on the milk production, or devaluation of the animals because of early elimination of those presenting reproductive failure (Thurmond and Hietala, 1997b; Trees *et al.*, 1999). It is noteworthy that economic losses seem to be more relevant in dairy cattle, due to higher abortion risk (Moore *et al.*, 2009). In a review carried out by Reichel *et al.* (2013) about economic impact of bovine neosporosis, it was estimated in one billion

dollars per year, and since this study is restricted to several of the most important countries on cattle population (10 countries), it is thought that the economic impact could be substantially greater.

3.7. Diagnosis

Diagnosis of bovine neosporosis is not easy and should be addressed in a proper and systematic way. Clinical and epidemiological information may suggest *N. caninum* infection and its involvement in the abortion. However the definitive diagnostic always need to be confirmed by laboratory techniques. Selection of the most adequate tool in each case, as well as its correct interpretation, play an essential role (Ortega-Mora *et al.*, 2006). In this way, individual diagnosis to determine the abortion cause in an animal or the presence of infection in replacement heifers can be performed. Also, diagnosis at herd level can be evaluated when attempting to determine infection in the herd, involvement of *N. caninum* in reproductive problems, as well as the main mode of transmission, in order to stablish appropriate control measures. Nowadays, there is a wide a range of diagnostic techniques, but not all provided the same information or have the same accuracy. However, since animals are chronically infected with *N. caninum*, the most useful diagnostic techniques in live animals are based on detection of specific *N. caninum* antibodies in serum. To date, different techniques for serological diagnosis have been established, such as IFAT, agglutination and immunoblot, being the ELISA the most commonly used (Alvarez-Garcia *et al.*, 2013). ELISA test can be used in adult animals and newborn animals, when precolostral serum is utilized. Furthermore, avidity ELISAs capable of differentiate recent and chronic infections are available (Schares *et al.*, 2002; Aguado-Martínez *et al.*, 2005; Bjorkman *et al.*, 2006) and ELISAs based on NcGRA7 and NcSAG4 proteins (tachyzoite -acute phase- and bradyzoite -chronic phase- specific proteins, respectively), allow the differentiation between primoinfection, recrudescence and chronic infection (Aguado-Martínez *et al.*, 2008). Likewise, ELISA in milk samples has been achieved, exhibiting comparable results with ELISA from sera, with the added advantages of lower costs and animal management (Bjorkman *et al.*, 1997; Schares *et al.*, 2004; Schares *et al.*, 2005; Hall *et al.*, 2006).

The serological status in the herd and aborted cows allows to determine if abortions are associated to *N. caninum* infection and its extent. This first serological approach must be accompanied with techniques for parasite detection (PCR) and evaluation of compatible lesions in tissues from aborted foetuses (mainly brain) (Buxton, 1998; Dubey and Schares, 2006; Ortega-Mora *et al.*, 2006). In addition, despite the limited value of serology as diagnostic tool in aborted foetuses, the detection of specific antibodies in fluids from foetuses above 5 months of gestation can complement the diagnosis, since in ruminants, antibodies are not able to cross the placental barrier and therefore, antibodies are produced by the foetus (Pereira-Bueno *et al.*, 2003; Dubey and Schares, 2006; Ortega-Mora *et al.*, 2006).

Once *N. caninum* infection and herd serological status are confirmed, and in order to implement the most appropriate control measures, is important to determine the pattern of abortions, as well as the main mode of transmission in the herd. In this sense, relation between maternal and foetal serology, *odds ratio* (OR) calculation, evaluation of avidity antibodies in aborted animals and the age distribution of seropositive animals, can guide about the main mode of transmission in the herd. The OR evaluates the risk of abortion in seropositive animals, while avidity tests determine the antiquity of infection in the herd. Thus, epidemic abortions associated to horizontal transmission are characterized by OR>2, low avidity antibodies, heterogeneous age distribution of abortions and lack of association between seropositivity of dam and its offspring. By contrast, endemic abortions typical after recrudescence in chronically infected herds, exhibit OR<2, high avidity antibodies,

homogeneous age distribution of abortions and association between serology in dam and its offspring (Dubey *et al.*, 2007).

4. Control of toxoplasmosis and neosporosis in ruminants

For the control of toxoplasmosis and neosporosis different measures have been suggested, including management practices, chemotherapy and vaccination, however, the combination of different approaches is known to be the optimal strategy (Dubey *et al.*, 2007; Dubey, 2009b). For ovine toxoplasmosis there is a live vaccine available, while no vaccine is currently in the market for bovine neosporosis. Likewise, there is lack of registered drugs for the treatment of toxoplasmosis and neosporosis in ruminants. In the case of high prevalence of the neosporosis, drugs and vaccination are considered the best strategies from an economic point of view to avoid reactivation (Reichel and Ellis, 2006). However, in toxoplasmosis, drugs and vaccination should be preferable applied if there is a likely environmental contamination with oocysts or after an abortion outbreak to avoid the clinical manifestation of the disease.

4.1. Control of the transmission

The implementation of farm biosecurity protocols, hygienic measures and management practices should be adopted in all farms, for reducing the level of environmental contamination with *T. gondii* oocysts via cat faeces or *N. caninum* oocysts via dog faeces (if present) and for avoiding novel infections through the introduction of infected animals to the herd. Among the biosafety practices that should be conducted to achieve this aim, the following are highlighted: limit cat and dog access to ruminant areas, especially to those housing pregnant ruminants, or to the areas for food storage and water supplies; promptly remove of placentas or foetal materials; appropriately dispose of dead livestock; and establish rodent control. Reproductive practices considering the artificial insemination of seropositive dams or the use of embryo transfer and test and cull strategies based on serological diagnosis are also recommended for the control of *N. caninum* infections (Dubey *et al.*, 2007; Reichel *et al.*, 2014; McAllister, 2016). Despite being properly designed and meticulously practised, globally, these control measures alone are not cost-viable or completely effective in eliminating toxoplasmosis and neosporosis from a herd, and it is necessary to complement them with an immune-chemotherapeutical approach (Zhang *et al.*, 2013; Hemphill *et al.*, 2016; McAllister, 2016).

4.2. Treatment

4.2.1. Available drug treatments for ruminant toxoplasmosis and neosporosis

In this section the efficacy of drugs against *T. gondii* and *N. caninum* in ruminants is presented. Efficacy of this drugs *in vitro* and in mice models can be found in Appendix 1.

4.2.1.1. Macrolide antibiotics

Spiramycin has been tested in pregnant ewes infected with *T. gondii* (RH strain) between the 85th and 100th days of pregnancy and treated orally (PO) with 100 mg/kg spiramycin from 3 weeks after infection until parturition. All ewes gave birth, with only one stillbirth in the untreated group. Analysis of the placental tissues did not show differences neither in the histopathological lesions, nor in the presence of *T. gondii* between the treated and untreated groups. However, the humoral immune response in pregnant ewes decreased in the treated group compared to that in the untreated group.

Additionally, a lower number of lambs were seropositive to *T. gondii* in the treated group than in the untreated one. It was concluded that spiramycin treatment in ewes during the mid-stage of gestation exhibited a reduction in the humoral immune response in dams and in *T. gondii* seropositive lambs (Dubreuil, 1972).

4.2.1.2. Polyether ionophore antibiotics

The efficacy of monensin against ovine toxoplasmosis was evaluated in experimentally infected pregnant sheep. Ninety-days pregnant ewes were experimentally infected PO with 2,000 and 12,000 *T. gondii* (M1 strain) oocysts and were treated PO with monensin (15 or 30 mg) daily from day 80 of gestation until parturition (Buxton *et al.*, 1988). Ewes receiving monensin showed a reduction in foetal mortality compared with the non-treated ewes (83.3% *vs* 44.8% alive lambs, respectively). In this study, monensin seems to act earlier in the infection, possibly by effects on the sporozoites released from infectious oocysts within the intestinal lumen. Ewes infected and treated with monensin showed a lesser febrile response. In addition, ewes receiving monensin showed lower anti-*T. gondii* IgG levels. However, when monensin administration ceased after lambing, circulating specific IgG antibodies against *T. gondii* increased over three months to reach values similar to those observed in infected and non-treated ewes. This observation suggests that monensin could also have a systemic effect, possibly acting on the tachyzoites present in the pregnant uterus. In addition, lambs born from ewes receiving monensin had higher live weights, and less of these animals showed evidence of infection and pathological changes in foetal or placental tissues than the lambs born from infected and non-treated ewes.

The possible systemic effect of monensin after *T. gondii* infection could be of value, as the time of infection during natural outbreaks of toxoplasmosis can only rarely be clearly defined. To avoid intestinal infection, and hence any intestinal effect of monensin PO at day 90 of pregnancy, the pregnant ewes were challenged subcutaneously (SC) with 100 *T. gondii* (M1 strain) tissue cysts (Buxton *et al.*, 1987). No differences in febrile and serological responses were found between the treated and non-treated groups. However, the treated ewes produced more viable lambs (less premature and greater live weight) than the untreated ewes (75% *vs* 42%, respectively). In addition, 58% of the lambs from treated ewes survived 72 hours after birth, whereas only 33% survived from the untreated ewes. Based on this evidence, it would appear that monensin, while being most effective in the gut lumen, does have a lesser systemic action, presumably by suppressing the multiplication of *T. gondii* in the placentome.

The accidental poisoning of domestic animals with monensin PO has been reported in cattle, horses, poultry and dogs (Beck and Harries, 1979; Wilson, 1980). The clinical findings in sheep include lethargy, stiffness, muscular weakness, a stilted gait and recumbency, followed by a decrease in the muscle volume of the rump and thigh. The post-mortem lesions in the skeletal muscles consisted of pale streaking, with atrophy in the chronic stages. In lambs younger than one month old, diffuse gastrointestinal haemorrhage was the only finding (Nation *et al.*, 1982). Later, another researcher reported an outbreak of monensin poisoning in sheep and highlighted the need for a licensed, safe product (Synge, 1989). An option to avoid monensin poisoning in sheep would be to use blocks containing monensin or slow-release boluses. In addition, this option would overcome the practical problems of monensin delivery to grazing sheep (Trees, 1989; Ellis and Costigan, 1990).

Lasalocid has also been tested for its efficacy against ovine toxoplasmosis (Kirkbride *et al.*, 1992). Ewes were treated PO with lasalocid (30 g/day) daily from day 55 of pregnancy until lambing and were PO inoculated with 100 *T. gondii* (TS-1 strain) oocysts 5 days after beginning lasalocid administration. Similar specific antibody titres and histopathological lesions were found in the ewes

and foetuses, and there were no differences in the rate of abortion and neonatal mortality in both the treated and untreated ewes. These results suggest that lasalocid was not effective in preventing *T. gondii* abortion in sheep.

Concerning *N. caninum* infection in cattle, a risk factor analysis in dairy farms showed that cows receiving monensin as a feed additive were 1.5 times less likely to be infected with this parasite than the cows that did not receive monensin (Vanleeuwen *et al.*, 2010). Thus, the effect of monensin was tested against experimental neosporosis in cattle (Vanleeuwen *et al.*, 2011). Non-pregnant cows were treated with a slow-release bolus PO that delivered 100 days of monensin (335 mg/day) and were challenged with $5x10^6$ *N. caninum* (Nc-1 strain) tachyzoites by the SC route three weeks after bolus administration. The cows treated with monensin showed a significantly lower humoral immune response than those treated with a placebo at week 4 post-challenge. Before recommendations on monensin use for neosporosis could be made, further research on other larger populations of cattle, preferably pregnant, must be explored. Furthermore, trials on monensin's effectiveness in more natural modes of *N. caninum* transmission (e.g., bradyzoite recrudescence leading to transplacental transmission, or oocyst ingestion) would also be needed.

In summary, PO administered monensin was shown to be partially effective in the control of ovine toxoplasmosis, but it is not licensed for use in the EU and may be toxic at high dosages. In contrast, PO dosed lasalocid did not exhibit efficacy in reducing the ovine toxoplasmosis outcome. In *N. caninum*-infected non-pregnant cows, monensin was associated with a lower specific humoural immune response, but further research on the outcome of infection in pregnant animals is needed.

4.2.1.3. Folate inhibitors

Experimentally induced toxoplasmosis in pregnant ewes with 2,000 oocysts (M3 strain) at 89 days of pregnancy was treated with a combination of sulphamezathine and pyrimethamine sulphate for three consecutive days on days 100, 115 and 130 of gestation. Sulphamezathine (1 g per 3 ml of solution) was injected SC at an initial dose of 5 ml/10 kg on the first day, with subsequent doses on the following two days of 2.5 ml/10 kg. Pyrimethamine sulphate (10 mg/ml) was injected IP at 2 mg/kg on the first treatment day and at 1 mg/kg on the two subsequent days. After 130 days of gestation, 30% of foetuses from untreated ewes died, whereas all the lambs from the treated ewes were born and were viable. In addition, the gestational period of infected and untreated ewes was shorter than that observed in the infected and treated ewes. At birth, all the lambs showed a specific antibody response to the protozoa, indicating that the infection was in utero. However, the mean IFAT titres of lambs born from the infected and treated ewes were lower than those of the lambs born from the infected and untreated ewes (1/256 *vs* 1/730 for IgG and 1/665 vs 1/5206 for IgM). Histopathological examination showed less severe placental lesions in the lambs born from the treated ewes (Buxton *et al.*, 1993a).

In field conditions, the therapeutic efficacy of sulphonamides in an ovine toxoplasmosis outbreak has been evaluated. Before treatment, 60% of the ewes in the flock had aborted, and treatment with sulphadimine at 20 mg/kg intramuscularly (IM), 4 times every 48 hours, reduced the abortion rate to 25%; the rest of the ewes gave birth normally, but 61.1% of their lambs were stillbirths and did not survive. A higher dosage at 33 mg/kg IM, 4 times every 48 hours, reduced the abortion rate to 7%, and 75% of the lambs survived (Giadinis *et al.*, 2011). Folate inhibitors have also been evaluated in field conditions for the treatment of neosporosis in cattle. A combination of toltrazuril given intravenously (IV) at 20 mg/kg/day for 3 consecutive days to newborn calves during the first week of age and sulphadiazine/trimethoprim given IV at 20 mg/kg to cattle once a year from 3 months of age

along with dog treatment with toltrazuril and periodic disinfection of environment resulted in a significant reduction of abortion (from 188 to 9) (Cuteri *et al.*, 2005).

In brief, parenteral administration of sulphonamides, in combination with pyrimethamine, could be a valuable option for chemotherapy of ovine toxoplasmosis in the third term of gestation, as a reduction in abortion rates, but not in the percentage of transplacental transmission, is observed. There is some evidence in field experiments that folate inhibitor administration can reduce abortions in ruminants, although more research is needed.

4.2.1.4. Quinolones

In a study, sheep were challenged PO with 200 *T. gondii* (M3 strain) oocysts at 90 days of gestation, and decoquinate was administered PO at 1 or 2 mg⁄kg⁄day from 10 days prior to oocyst challenge until lambing (Buxton *et al.*, 1996). The administration of decoquinate at the higher rate of 2 mg⁄kg⁄day was associated with a delayed onset of the febrile response to infection, reduction in the overall severity of fever and a delay in the production of specific antibodies to the parasite. This treatment also reduced the placental damage caused by the protozoa, lengthened the mean gestation period by five days and increased the proportion of viable lambs (up 61.8%) and the mean weight of the lambs (up 33.3%) in comparison with ewes that were not treated with decoquinate but were challenged with *T. gondii* oocysts.

In addition, it is necessary to note that the decoquinate-medicated feed distributed to pregnant heifers that are chronically infected or primo-infected with *N. caninum* at the dose of 2 mg/kg from 1.5 months of gestation until the end of the 8th month of pregnancy tends to reduce the associated abortions (from 38% to 21% in chronically infected heifers and from 17% to 6% in primo-infected heifers) and neonatal infections, as more seronegative calves were born (28% in the treated group *vs* 21% in the untreated group for chronically infected heifers and 59% in the treated group *vs* 35% in the untreated group for primo-infected heifers) (Journel *et al*., 2002)

To summarize, PO administration of decoquinate reduces the effect of experimentally induced toxoplasmosis in pregnant ewes. These results support the indication of decoquinate as an aid in the prevention of abortion due to ovine toxoplasmosis if used during mid and late pregnancy. Decoquinate may have an application in the treatment of bovine neosporosis.

4.2.1.5. Triazinones

It has been emphasized that the production of *T. gondii* free lamb, sheep or goat meat for human consumption is critically important for public health (Kijlstra and Jongert, 2009). The *in vivo* therapeutic efficacy of toltrazuril on *T. gondii* tissue cysts in experimentally infected lambs has been studied after a chronic infection in newborn lambs through a parenteral inoculation of 10^5 *T. gondii* (ME-49 strain) oocysts (Kul *et al.*, 2013). Beginning at the 15th day after inoculation, the lambs were treated with toltrazuril PO 2 times, once every week at a dose of 20 mg/kg and 40 mg/kg. Following toltrazuril treatment, at day 90 after inoculation, the specific immune humoral response in lambs of both treatment groups was lower. On day 90 after inoculation, the lambs were necropsied. The histopathological findings in the toltrazuril-treated lambs include morphologic and structural changes of the tissue cysts in the musculature, which were characterized by initial degenerative changes in the cyst wall and a minimal inflammatory cell response. The presence of *T. gondii* DNA in heart, brain and semitendinosus muscle from the treated groups was lower than that in tissues of the non-treated lambs. Moreover, in the treated groups, 4 out of 9 (44.4%) lambs did not contain any tissue cysts in the examined tissues, but untreated animals showed *T. gondii* tissue cysts at least in one of the sampled

muscles. The administration of toltrazuril seems to be associated with a reduction of *T. gondii* cysts in the musculature.

It is doubtful that encysted *N. caninum* bradyzoites were susceptible to toltrazuril treatment. Congenitally infected calves born from *Neospora*-seropositive cows were PO treated with toltrazuril at 20 mg/kg three times at 48-hour intervals within 7 days after birth, following a humoral immune response (Haerdi *et al.*, 2006). Four to six months after birth, a stronger antibody reactivity was found in the treated animals than in the untreated calves. Conversely, in a study to evaluate whether the treatment of congenitally infected lambs with toltrazuril PO at 20 mg/kg on days 0, 7, 14 and 21 after birth eliminated *N. caninum*, toltrazuril did not show any effect on the reduction of *N. caninum* presence or on the severity of histopathological lesions, and the lambs were all seropositive, although they had significantly lower specific antibody levels than those in the untreated animals, suggesting higher antigenic stimulation in the non-treated lambs than in the treated lambs (Syed-Hussain *et al.*, 2015a).

The efficacy of ponazuril has been tested in calves that were experimentally infected with *N. caninum* at $2x10^8$ tachyzoites (Nc-1 strain) (Kritzner *et al.*, 2002). Medication was performed PO with 20 mg/kg of ponazuril. The first medication dose was applied 24 hours after infection, and if repeated, it was administered every subsequent 24 hours for six days. Ponazuril allows a complete abrogation of parasite DNA detection by PCR in the brain and other organs. Regarding the non-medicated calves, it is noteworthy that there was a relatively lower susceptibility of calves to experimental infection, since only 50% of the calves became PCR-positive in the brain and muscles. The efficacy of a six-day treatment was also suggested by the significantly lower anti-*N. caninum* antibody response and later seroconversion than those in the infected and non-medicated calves.

In addition, as explained above, toltrazuril administration to newborn calves, along with sulphadiazine/trimethoprim administration, resulted in reduced abortions and *N. caninum* seroprevalence in naturally infected cattle herds (Cuteri *et al.*, 2005).

In summary, toltrazuril PO administered in lambs would be a valuable strategy to minimize human exposure to *T. gondii* tissue cysts from the consumption of raw or undercooked mutton. Triazinon derivatives are directed against an *N. caninum* tachyzoite challenge, whereas treatment of congenitally infected young ruminants remains an elusive goal. In addition, it would likely result in considerable unacceptable milk or meat residues or withdrawal periods (Dubey *et al.*, 2007).

4.2.2. Present approaches in drug development for ruminant toxoplasmosis and neosporosis

Experimental studies have revealed that several compounds have potentially interesting effects on *T. gondii* and *N. caninum in vitro*, but only a few drugs have been tested in laboratory animal models *in vivo*. The most interesting drugs tested for toxoplasmosis and neosporosis were derived from screenings in other intracellular protozoan parasites, including *Plasmodium*, *Trypanosoma* and *Leishmania* species, and some drugs exhibited broad-spectrum anti-parasitic activity against various protozoan and helminth parasites. However, it is notable that *T. gondii* was the least responsive to these sets of drugs, suggesting that it may be more difficult to target chemotherapeutically than the aforementioned parasites (Guiguemde *et al.*, 2010). In addition, other approaches identified compounds that inhibited targets that were conserved almost exclusively within the group of apicomplexan parasites; thus, drug repurposing is a valuable option (Brown and Superti-Furga, 2003; Hemphill *et al.*, 2016). The main prospective drug classes with *in vitro* and *in vivo* activity in small animal models against *T. gondii* and *N. caninum* are reviewed below, although more

chemotherapeutic options for toxoplasmosis and neosporosis have been described (Neville *et al.*, 2015; Hemphill *et al.*, 2016).

4.2.2.1. Thiazolides

Nitazoxanide (2-acetolyloxy-N-(5-nitro 2-thiazolyl) benzamide), the mother compound of this class, is essentially composed of a nitrothiazole-ring and a salicylic acid moiety, which are linked together through an amide bond (Hemphill *et al.*, 2006; Hemphill *et al.*, 2007). Since nitazoxanide non-selectivity can lead to undesired side effects in both human and animals, nitazoxanide derivatives were designed without the undesirable nitro group (Esposito *et al.*, 2007b). The nitazoxanide derivative RM4847 produces the upregulation of NQO1 (quinone reductase) expression by *N. caninum* but not by *T. gondii*. This fact may reflect differences between these parasites in terms of the mechanisms of circumventing host cell apoptosis (Müller and Hemphill, 2011). Thiazolides have favourable effects against *N. caninum in vitro*, with an IC_{50} of 4.23 µM and 13.68 µM for nitazoxanide and RM4847, respectively. Host cell invasion of extracellular *N. caninum* tachyzoites is inhibited by RM4847, but not by nitazoxanide (Esposito *et al.*, 2007a), and RM4847 was shown to be much more effective against *T. gondii* tachyzoites, with an IC_{50} of 0.2 µM (Müller *et al.*, 2009). For the treatment of *N. caninum* in mice, nitazoxanide fails when it is applied PO, or it is even toxic when applied IP (Debache *et al.*, 2011). In calves, the use of nitazoxanide to treat *Cryptosporidium* infection showed acute diarrhoea in non-infected animals, which indicates that this compound severely affects the intestinal flora (Schnyder *et al.*, 2009). In conclusion, thiazolides might be interesting tools to study the biology of *N. caninum* and possibly *T. gondii*, but they are useless as drugs since they exert acute toxicity in small and large animals.

4.2.2.2. Diamidines

Diamidines represent a class of broad-spectrum antimicrobial compounds in which pentamidine and its analogues exhibit activity against intracellular and extracellular protozoan parasites (Wilson *et al.*, 2008; Buckner and Navabi, 2010). Pentamidine displays *in vitro* activity against *T. gondii* by inhibiting the replication of the parasites in cell cultures (Lindsay *et al.*, 1991).

Pentamidine derivatives, and the more recently synthesised di-cationic arylimidamides, which have more favourable pharmacokinetic profiles, improved bioavailability, lowered host toxicity and had a higher chance of passing the blood-brain barrier to exert its activity by binding to AT-rich sites in the DNA minor groove, thus inhibiting transcription or the interaction with DNA-binding enzymes, such as topoisomerases or nucleases (Wilson *et al.*, 2008). This result indicates that these compounds could influence gene expression, and thus many diverse cellular functions could be affected. Di-cationic arylimidamides, such as DB786, DB750 and DB745, showed *in vitro* activity against *T. gondii* tachyzoites, with an IC_{50} of 0.22 µM, 0.16 µM and 0.03 µM, respectively (Leepin *et al.*, 2008; Kropf *et al.*, 2012). In contrast to DB750, DB745 also had a profound negative impact on extracellular *T. gondii* tachyzoites. In addition, a lower adaptation of *T. gondii* tachyzoites to DB745 was observed (Kropf *et al.*, 2012). The lowest IC_{50}s against *N. caninum* tachyzoites were found for DB786, DB750 and DB745 (0.21 µM, 0.23 µM, 0.08 µM, respectively), which caused damage to the parasite's ultrastructure. The activities of DB750 and DB786 are limited to intracellular *N. caninum* tachyzoites; however, DB745 had an impact on both host cell invasion and intracellular proliferation (Leepin *et al.*, 2008; Schorer *et al.*, 2012). Di-cationic arylimidamides have also been found to be effective against neosporosis in mice. DB750 that was administered IP prior to infection or 14 days after infection reduced the severity of clinical signs and the cerebral parasite load, while PO application resulted in weight loss, indicating toxicity (Debache *et al.*, 2011; Schorer *et al.*, 2012). In addition, DB745 IP treatment initiated 14 days after infection had similar positive effects on the percentage of

surviving mice and the parasite load in the brain (Schorer *et al.*, 2012). Arylimidamide treatments in mice beginning 14 days post-infection, after the *N. caninum* tachyzoites had crossed the blood-brain border and invaded the central nervous system (CNS) (Collantes-Fernández *et al.*, 2006a), indicated that DB745, just like DB750, most likely crossed the blood–brain barrier and also exerted its action within the cerebral tissues (Debache *et al.*, 2011; Schorer *et al.*, 2012). Potentially, features of DB745 could open the door for testing this compound against neosporosis in ruminants.

4.2.2.3. Artemisinins

Chinese herbal extracts are thought to possess the desired properties of potency and low toxicity, and they show promise for the identification of new therapeutic agents. Artemisinin, a sesquiterpene lactone with an unusual endoperoxide bond in a unique 1,2,4-trioxane heterocycle [3R,5aS,6R,8aS,9R,12S,12aR)-octahydro-3,6,9-trimethyl-3,12-epoxy-12H-pyrano[4,3-j]-1,2-benzodioxepin-10(3H)-one] is present in the leaves and flowers of the sweet wormwood (*Artemisia annua*) (Bilia *et al.*, 2006), and its derivatives are highly potent antimalarial drugs. This safe drug class is also effective against apicomplexan parasites causing abortions in ruminants such as *T. gondii* and *N. caninum*. The mechanism of action of artemisinin and its derivatives is dependent upon the presence of the endoperoxide bridge (Haynes and Krishna, 2004), although the difference in potencies between *P. falciparum* and *T. gondii* suggests that different targets may be affected in these two organisms (Dunay *et al.*, 2009). Additionally, it is known that artemisinin perturbs calcium homeostasis in *T. gondii*, supporting the idea that Ca_2-ATPases (SERCA) are potential drug targets in parasites (Nagamune *et al.*, 2007). Artemisinin, when administered *in vitro* at 0.4 µg/ml for 5 days or 1.3 µg/ml for 14 days, completely eliminated *T. gondii* (Ke *et al.*, 1990). Artesunate showed an IC_{50} of 0.075 µM (Gomes *et al.*, 2012). Artesunate and its active metabolite, dihydroartemisinin, resulted in approximately 40% and 70% growth inhibition *in vitro*, respectively, and the combination resulted in approximately 65% inhibition. As they are able to cross the blood-brain barrier, *in vivo* experiments with low virulence *T. gondii* (DUR strain) challenge causing a chronic infection in mice, different from previous artemisinin derivatives studies with RH strain, showed a 40% reduction in the number of *T. gondii* tissue cysts found in the brain of mice treated 5 days with artesunate-dihydroartemisinin and also modifications in the microscopic aspect of the cysts (Sarciron *et al.*, 2000). Artemisone, a second-generation semi-synthetic artemisinin derivative, and artemiside, the thiomorpholine precursor of *artemisone*, with an improved half-live, oral bioavailability, metabolic stability in various animal systems (Haynes *et al.*, 2006), tolerance *in vivo* (Nagelschmitz *et al.*, 2008) and a lack of detectable neurotoxic potential (Nontprasert *et al.*, 1998; Schmuck *et al.*, 2009), are the most potent artemisinin analogues to date in terms of inhibiting the growth of *T. gondii in vitro*, with an IC_{50} of 0.12 µM and 0.10 µM for artemisone and artemiside, respectively. Both of these compounds (artemiside and artemisone) were effective in reducing mortality during an acute challenge (60% of artemiside-treated mice and more than 50% of artemisone-treated mice survived) and during the reactivation of chronic infection in a mouse model (80% of artemiside-treated mice and 60% of artemisone-treated mice). Furthermore, there was an accompanying reduction in the chronic burden of tissue cysts in the CNS (Dunay *et al.*, 2009).

Against *N. caninum*, artemisinin reduces the intracellular multiplication of tachyzoites at ≥0.1 µg/ml for 30 hours or 1 µg/ml for 14 days (Kim *et al.*, 2002), and artemether exhibited activity against tachyzoite replication with an IC_{50} of 1.0 µg/ml (Qian *et al.*, 2015). Additionally, artemisone, when added prior to infection or in established infection, reduced the number of *N. caninum*-infected cells (Mazuz *et al.*, 2012) with an IC_{50} of 3 nM, exerting their activity against intracellular parasites, causing ultrastructural alterations and switched aberrant gene expression, including bradyzoite markers (Muller *et al.*, 2015b). In a gerbil model for acute neosporosis, artemisone increased survival,

as 1 out of 8 mice died in the artemisone-treated group, while 8 out 9 mice succumbed in the control group (Mazuz *et al.*, 2012). However, in a mouse model of neosporosis, artemiside and artemisone had no effect on parasite loads in the brain or in the lungs (Müller *et al.*, 2016). In short, artemiside seems to be useful against *T. gondii in vitro* and in mice models whereas artemisone is less likely to be promising for neosporosis in ruminants.

4.2.2.4. Naphthoquinones

Naphthoquinone is a class of organic compounds derived from naphthalene. Buparvaquone (2-((4-tert-butylcyclohexyl)methyl)-3-hydroxy-1,4-naphthoquinone) and atovaquone (trans-2[4-(4-chlorophenyl) cyclohexyl]-3-hydroxy-1,4-naphthalenedione) are hydroxynaphthoquinones related to parvaquone and were originally developed as anti-malarial compounds (Hudson *et al.*, 1985; Hudson *et al.*, 1991).

Buparvaquone is currently commercially available for use in endemic regions against theileriosis in cattle (Wilkie *et al.*, 1998); however, in other regions of the world, e.g., the EU, it is not registered. A focus on the parasite mitochondrion as an anti-parasitic target has been a priority in the drug development field for decades (Mather and Vaidya, 2008; Sen and Majumder, 2008). Hydroxynaphthoquinones are structurally similar to the inner mitochondrial protein ubiquinone (also called coenzyme Q), which is an integral component of electron flow in aerobic respiration. Ubiquinone accepts electrons from the dehydrogenase enzymes and passes them to the electron transport cytochromes (Sun *et al.*, 1992). The passage of electrons from ubiquinone to the cytochrome *bc*1 (complex III) requires the binding of coenzyme Q complex III at the Qo cytochrome domain; it is this step that is inhibited by hydroxynaphthoquinones (Fry and Pudney, 1992; Pfefferkorn *et al.*, 1993; McFadden *et al.*, 2000). The consequence of this inhibition is the collapse of the mitochondrial membrane potential (Srivastava *et al.*, 1997). On the one hand, atovaquone inhibited the replication of *T. gondii* tachyzoites *in vitro* with an IC_{50} of 64 nM (Romand *et al.*, 1993; Meneceur *et al.*, 2008). Moreover, atovaquone is active *in vitro* against the cyst stage of *T. gondii* (Huskinson-Mark *et al.*, 1991), although high concentrations of it are required (Araujo *et al.*, 1991; Araujo *et al.*, 1992). In a mouse model of acute toxoplasmosis, atovaquone administration resulted in prolonged survival, with a reduction of parasite burdens in the blood and tissues during the course of treatment (Araujo *et al.*, 1991; Romand *et al.*, 1993). In a mouse model of chronic toxoplasmosis, atovaquone showed a decline in the number of tissue cysts and a decrease in the inflammatory response in the brain (Araujo *et al.*, 1991; Araujo *et al.*, 1992; Ferguson *et al.*, 1994). Indeed, atovaquone is effective against reactivation, as it protected mice against reactivated toxoplasmic encephalitis (Dunay *et al.*, 2004).

In short term studies, buparvaquone efficiently inhibited the replication of *N. caninum* tachyzoites with an IC_{50} of 4.9 nM exerting parasiticidal activity after 9 days of culture in 0.5 µM or 6 days in 1 µM buparvaquone. However, in the long-term studies, the tachyzoites reached an adaption to high levels of buparvaquone. Additionally, ultrastructural changes confirm that buparvaquone acted rather slowly (Muller *et al.*, 2015a). In a non-pregnant mouse model of neosporosis, buparvaquone reduced the mortality and parasite load in the lungs, while the brain parasite load was higher than in untreated mice (Muller *et al.*, 2015a); the brain parasite load was lower when a 20-times reduced challenge dose was used (Müller *et al.*, 2016). In addition, in a pregnant mouse model of neosporosis, pup mortality and the transmission of *N. caninum* to offspring were strongly reduced by buparvaquone treatment of the dams (Müller *et al.*, 2016). In light of these results, atovaquone represents a valuable candidate to be tested for toxoplasmosis in ruminants and, although further studies are required to improve the efficacy in a mouse model, buparvaquone represents an obvious candidate to be tested against neosporosis in ruminants.

4.2.2.5. Anticancer agents

Another example of prospective compounds that provide interesting results is anticancer drugs, as parasites have several of the common characteristics of malignant tumours (Klinkert and Heussler, 2006; Dissous and Grevelding, 2011; Dissous and Grevelding, 2011), sharing a crucial feature of living and multiplying in a host organism. Tumour cells are defined by their independence from exogenous growth factors, their resistance to programmed cell death (apoptosis), and their infinite proliferative capacity. Unlimited proliferation and independence of growth factors are also characteristics of many parasites. Although it remains controversial whether apoptosis occurs in unicellular parasites (Debrabant *et al.*, 2003), it seems to be clear that intracellular parasites interfere with the programmed host cell apoptosis (Lüder *et al.*, 2001). Parasite and cancer cells disseminate in immune compromised tissues in order to escape host immune responses. Anticancer drugs may affect parasite survival at two completely different levels. Firstly, they might kill the parasite directly, if the target molecules of parasite and cancer cell are sufficiently similar. In this case, the original cancer drugs may serve as leader compounds and can be modified accordingly to specifically inhibit the parasite homologue. Secondly, to kill intracellular parasites successfully, the drug might also act on a host cell signalling pathway, which is essential for the parasite's survival. The advantage here is that the drug need not be modified, since it is already directed against the target molecule (Klinkert and Heussler, 2006).

Miltefosine (2-[hexadecoxy-oxido-phosphinoyl] oxyethyl-trimethyl-ammonium), an alkyl phospholipid and an analogue of the ubiquitous compound phosphatidyl choline found in eukaryotic cell membranes, was initially developed as an anticancer agent (Wieder *et al.*, 1999) and widely used for *Leishmania* therapy (Solano-Gallego *et al.*, 2011). It is highly active against extracellular *T. gondii* tachyzoites and exerts its activity by triggering apoptosis (Nyoman and Lüder, 2013). Miltefosine has no effect on a mouse model of acute toxoplasmosis after 5 days of treatment, but it shows activity in a 15-day treatment against chronic experimental toxoplasmosis, with a 78% reduction in the brain cyst burden. Pathological findings showed that the tissue cysts were smaller in size upon microscopical examination and that there were ultrastructural changes in the remaining cysts, suggesting that miltefosine effectively penetrates the blood-brain barrier (Eissa *et al.*, 2015). Against *N. caninum*, miltefosine showed an IC_{50} of 5.2 µM *in vitro* with a parasitostatic effect at 25 µM for 10 hours and parasiticidal activity after 20 hours. In addition, *N. caninum* tachyzoites revealed ultrastructural changes after miltefosine exposure. In a mouse model of neosporosis, miltefosine improved mouse survival and reduced the cerebral parasite burden (Debache and Hemphill, 2012).

Recently, organometallic ruthenium complexes are object of great attention as antitumor agents with acceptable toxicity (Kostova, 2006; Bergamo and Sava, 2011). They also have antibacterial activity against some bacteria and parasites (Beckford *et al.*, 2011; Caroli *et al.*, 2012). Earlier studies indicated that ruthenium compounds interact with DNA (Schwietert and McCue, 1999), but more recent investigations showed that ruthenium compounds bind more strongly to proteins (Ravera *et al.*, 2004). Ruthenium compounds (compounds 16 and 18) were reported to exhibit IC_{50} values of 6–12 nM for *N.caninum* and 18-41 nM for *T.gondii* (Barna *et al.*, 2013). Furthermore, dinuclear thiolato-bridged arene ruthenium complexes (complex 9, complex 1 and complex 2) showed promising activities against *T. gondii* with IC_{50} values of 1.2 nM, 34 nM and 62 nM (Basto *et al.*, 2017). Therefore, anticancer drug such as miltefosine could be considered as an appropriate drug to be evaluated in ruminant model for toxoplasmosis and neosporosis whereas ruthenium complexes need to be evaluated in mice models before contemplate the option of testing in ruminants.

4.2.2.6. Endochin-like quinolones

Endochin is a 4-(1H)-quinolone initially investigated as an antimalarial drug (Salzer *et al.*, 1948). Subsequently, endochin was active against avian and murine toxoplasmosis (Gingrich and Darrow, 1951). Recent 4-(1H)-quinolone derivatives, endochin-like quinolones (ELQ), compounds that are analogs of ubiquinone, have been developed and tested against apicomplexan parasites such as *P. falciparum* and *T. gondii*, as well as other protozoa such as *Leishmania* parasites (Ortiz *et al.*, 2016). ELQs exert their activity by inhibit cytochrome c reduction by the cytochrome bc1 complex (Winter *et al.*, 2008; Doggett *et al.*, 2012). The cytochrome bc1 complex is a membrane-bound enzyme complex located in the inner mitochondrial membrane that contributes to pyrimidine biosynthesis and oxidative phosphorylation (Vercesi *et al.*, 1998). 3-alkyl-2-methyl-4(1*H*)-quinolones exhibited excellent *in vitro* activity against *P. falciparum* (Winter *et al.*, 2011). 4(1H)-quinolone-3-diarylethers, with improved stable and solubility properties, have been tested against *T. gondii* and ELQ-271 and ELQ-316 showed *in vitro* IC_{50} values of 0.1 nM and 0.007 nM, respectively. ELQ-271 and ELQ-316 were also efficacious against acute toxoplasmosis in mice when administered orally and against the cyst form of *T. gondii* in mice when administered intraperitoneally (Doggett *et al.*, 2012). Against *N. caninum*, ELQ-400 showed an IC_{50} value below 10 nM, had an impact on intracellular proliferation of tachyzoites and transmission electron microscopy showed that the primary target of ELQ-400 was the mitochondrion. In experimentally infected non-pregnant mice, ELQ-400 orally showed a reduction in the number of animals with lung and brain infection, as well as a reduction in the humoral immune response against *N. caninum* (Müller *et al.*, 2017a). Thus, ELQ-316 is a promising starting point for the development of a future toxoplasmosis therapy in ruminants, and ELQ-400 showed hopeful results against *N. caninum*, but further studies are needed to assess efficacy in pregnant animal models.

4.2.2.7. Calcium-dependent protein kinase inhibitors

Calcium signalling is a very important pathway that regulates diverse cellular processes (Berridge *et al.*, 2000). In apicomplexan parasites, this signalling pathway directs motility, cell invasion and egression (Lourido *et al.*, 2012). Members of the family of calcium dependent protein kinases (CDPK's) are abundant in certain pathogenic parasites and absent in mammalian cells making them strong drug target candidates. Genetic disruption of CDPKs has shown they control a wide range of phenotypes in *T. gondii* including egress (TgCDPK1 and TgCDPK3) (Lourido *et al.*, 2010; Garrison *et al.*, 2012; Lourido *et al.*, 2012; McCoy *et al.*, 2012), microneme secretion (TgCDPK1) (Lourido *et al.*, 2012), motility (TgCDPK1) (Lourido *et al.*, 2010), or cell division (TgCDPK7) (Morlon-Guyot *et al.*, 2014). Other CDPKs in *T. gondii* (CDPK4, CDPK4A, CDPK5, CDPK6, CDPK8, and CDPK9) were described non essential for the lytic cycle (Wang *et al.*, 2016). CDPKs may are also involved in other functions in *T. gondii*, for example, CDPK2 is essential for bradyzoite development (Uboldi *et al.*, 2015) and CDPK4A, CDPK6 and CDPK7A may play roles during sporozoite formation or transmission via oocysts (Long *et al.*, 2016).

Bumped kinase inhibitors (BKIs), a particular class of pyrazolopyrimidine inhibitors of CDPK1, have bulky C3 aryl moieties entering a hydrophobic pocket in the ATP binding site. BKIs selectively inhibit CDPK1 from apicomplexans in a good structure-activity relationship (Keyloun *et al.*, 2014), but they do not inhibit mammalian kinases because they have larger amino acid residues adjacent to the hydrophobic pocket, thereby blocking the entry of the bulky C3 aryl group. CDPK1 is found in most apicomplexans, and the highly conserved nature of the ATP binding domain shared by apicomplexan CDPK homologues could be exploited, to some extent, in the development of potential broad-spectrum inhibitors (Keyloun *et al.*, 2014). BKIs were originally developed to combat malaria (Ojo *et al.*, 2012), but they have been tested for many apicomplexan parasites (Van Voorhis *et al.*,

2017). BKI-1294 acted with an IC_{50} of 20-220 nM *in vitro* for different strains of *T. gondii* (Winzer *et al.*, 2015). BKI-1294 was highly effective against acute toxoplasmosis in mice, decreasing the numbers of *T. gondii* tachyzoites in the peritoneal lavage fluid (Doggett *et al.*, 2014) and in a murine vertical transmission model of *T. gondii (*Müller *et al.*, 2017c*)*.

BKI-1517 showed a 3-times lower IC_{50} than BKI-1553 and BKI-1294 against *N. caninum in vitro* (Ojo *et al.*, 2014; Müller *et al.*, 2017b). In a pregnant mouse model, BKI-1294, BKI-1517 and, less clearly, BKI-1553 achieved protection against the vertical transmission of *N. caninum*, but BKI-1553 and more markedly BKI-1517 showed detrimental effects on fertility (Winzer *et al.*, 2015; Müller *et al.*, 2017b). Hence, BKI-1294 could be considered a promising drug to be tested in ruminant models for toxoplasmosis and neosporosis.

Since BKIs showed parasitostatic rather than parasiticidal effects, they are appropriated for a combined immunization plus treatment protocol. These compounds triggered the formation of relatively long-lived multinucleated complexes where parasites are blocked in the process of cytokinesis and remain trapped within the host cell but are still viable. These multinucleated complexes express specific antigen SAG1 and the bradyzoite marker BAG1 (Winzer *et al.*, 2015; Müller *et al.*, 2017b). Thereby, these antigens may be presented to the immune system eliciting stable immune responses against tachyzoite as well as bradyzoite stages.

Apart from CDPK1, the rarity of kinases containing small gatekeeper residues in the apicomplexan genome reduces the chance of off-target effects, although intermediate sensitivity is expected for kinases containing A, S, or T in the gatekeeper position, thus potentially limiting this approach in some cases. However, an interesting strategy based on the exploitation of the kinase gatekeeper residue was recently developed to identify parasite CDPKs substrates (Lourido *et al.*, 2013).

4.3. Vaccination

4.3.1. *T. gondii* vaccines

The role of a vaccine against *T. gondii* would be to prevent parasitaemia and colonization of placenta and ideally, prevent formation of tissue cysts which are a source of infection to humans (Menzies, 2012). In mice a large number of vaccines based on inactivated antigens, live attenuated vaccines, vaccines based on recombinant proteins, DNA vaccines and vector-based vaccines have been evaluated (Jongert *et al.*, 2009).

In sheep, several *T. gondii* vaccines have been also assessed (Hiszczyńska-sawicka *et al.*, 2014). The commercial attenuated live vaccine, Toxovax® (MSD) was designed to limit the incidence of abortion in sheep related to *T. gondii*. It results in a decrease of abortion but does not completely eradicate the parasite (Buxton and Innes, 1995). This vaccine is based on the S48 isolate, which was originally isolated in New Zealand from an aborted lamb and after over 3000 passages in mice it lost the ability to form tissue cysts or oocysts (Buxton *et al.*, 1991; Buxton and Innes, 1995). Following subcutaneous inoculation of naïve sheep, the parasite multiplies in the local draining lymph node, causing a mild febrile response with peak titres of antibody reached by 6 weeks. The immunity induced by this vaccine is likely to involve both CD4+ and CD8+ T cells and IFN-γ (Montoya and Liesenfeld, 2004). Vaccination induces immunity that protects against abortion for at least 18 months after the initial vaccination (O'Connell *et al.*, 1988; Wilkins *et al.*, 1988; Buxton *et al.*, 1993b). This vaccine is only licensed for sheep and the administration of a single shot prior to mating is recommended.

An attenuated *T. gondii* isolate was also evaluated as a vaccine to prevent the infection of meat animals. It is shown that lambs immunized with oocysts of the relatively avirulent TgME49 isolate, and then challenged with a virulent M3 isolate, developed complete prevention of the formation of virulent M3 cysts in lamb muscle (Falcon and Freyre, 2009). Another study assessed the effectiveness of a mutant isolate lacking the MIC1 and MIC3 genes (Mévélec *et al.*, 2010). Ewes inoculated subcutaneously or intraperitoneally, 2 months before mating, with MIC1-3KO tachyzoites developed a mild febrile response and serum IgG antibodies, which persisted throughout the experiment. Pregnant ewes were challenged orally with sporulated oocysts. All unvaccinated ewes aborted, whereas 62-91% of the lambs from vaccinated ewes were viable (Mévélec *et al.*, 2010). Moreover, subcutaneous vaccination with MIC1-3KO tachyzoites showed potential for reduction in the development of tissue cysts in lambs born to vaccinated ewes. These results are encouraging in respect to the prevention of parasite transmission from food animals to humans.

Only an increase on the humoral and/or cellular immune responses have been elicited in sheep by vaccines based on tachyzoite antigens (Lundén, 1995; Stanley *et al.*, 2004), vector vaccines expressing GRA1 (Supply *et al.*, 1999) and DNA vaccines encoding GRA, ROP, MAG and MIC proteins (Hiszczynska-Sawicka *et al.*, 2010; Hiszczyńska-Sawicka *et al.*, 2010; Hiszczyńska-Sawicka *et al.*, 2011a; Hiszczyńska-Sawicka *et al.*, 2011b; Hiszczynska-Sawicka *et al.*, 2012). However, immune responses induced by DNA vaccines have not yet been evaluated for their ability to provide a protective effect against abortion in pregnant ewes.

4.3.2. *N. caninum* vaccines

An ideal *N. caninum* vaccine should prevent abortion and vertical transmission of the parasite. For several years, a commercial killed vaccine against bovine neosporosis based on a tachyzoite lysate (Bovilis Neoguard) was available in some countries (Barling *et al.*, 2003). This vaccine exhibited a moderate protection under field conditions, with a reduction of 50% in the abortion (Romero *et al.*, 2004). However, recent studies have revealed large differences in the efficacy at farm level, showing certain degree of protection against horizontal transmission, but no effect on vertical transmission, being even suggested that vaccination increased the risk of early foetal death in immunized cows (Weston *et al.*, 2012). At present, this vaccine has been withdrawal from the market and, therefore, the effort of numerous research groups is focused on the identification of new vaccine targets that allow the development of safe and effective vaccines against bovine neosporosis.

The most promising results have been obtained with the use of live vaccines, due to their ability to stimulate the cellular immune response after a natural infection (Innes and Vermeulen, 2006). In fact, a high percentage of protection against vertical transmission in mice (Miller *et al.*, 2005; Ellis *et al.*, 2008; Marugán-Hernández *et al.*, 2011; Rojo-Montejo *et al.*, 2012) and cattle (Williams *et al.*, 2007; Hecker *et al.*, 2013; Rojo-Montejo *et al.*, 2013; Weber *et al.*, 2013) were found. The live vaccines tested include, the use of isolates attenuated *in vitro* by irradiation (Ramamoorthy *et al.*, 2006) or successive passages in cell culture (Bartley *et al.*, 2006; Bartley *et al.*, 2008), genetically modified isolates (Marugán-Hernández *et al.*, 2011) or natural isolates with low virulence. Among the later, there are low-virulence isolates from asymptomatic animals congenitally infected such as the Nc-Nowra isolate (Miller *et al.*, 2002) and the Nc-Spain1H isolate (Rojo-Montejo *et al.*, 2009). However, in spite of the good efficacy results (Rojo-Montejo *et al.*, 2013), there are no live vaccines commercially available due to the difficulties on large-scale production and the lack of viability in the medium-long term (Reichel *et al.*, 2015). Additionally, in the live vaccines, especially in those using attenuated parasites, the risk of a possible reversion to virulence is present. Therefore, there is a safety problem in immunized animals.

Thus, the use of inactivated vaccines arise as an alternative to the live vaccines in terms of safety, cost and stability of the final formulation and killed tachyzoites and antigen extracts have been used. However, most of the studies evaluating inactivated showed negative or ambiguous results in the protection against *N. caninum* infection (Liddell *et al.*, 1999a; Cho *et al.*, 2005; Ribeiro *et al.*, 2009; Innes *et al.*, 2011; Mansilla *et al.*, 2013). This could be due to the lower stimulation of the protective immune responses, since there is no intracellular replication of the parasite. This deficiency could be counteract with the administration of a booster and the choice of the correct adjuvant (Babiuk, 2002).

Finally, the study of new generation vaccines is another alternative in the long term, including DNA vaccines (Cannas *et al.*, 2003b; Liddell *et al.*, 2003; Jenkins *et al.*, 2004), vector vaccines (Nishikawa *et al.*, 2000; Nishikawa *et al.*, 2001; Ramamoorthy *et al.*, 2007) and mainly vaccines based on recombinant proteins such as NcSRS2 (Cannas *et al.*, 2003a; Vemulapalli *et al.*, 2007; Tuo *et al.*, 2011), MIC proteins (Cannas *et al.*, 2003b; Alaeddine *et al.*, 2005; Srinivasan *et al.*, 2007; Monney *et al.*, 2011), NcPDI protein (Debache and Hemphill, 2013) and the NcROP2 and NcROP40 proteins (Debache *et al.*, 2008; Debache *et al.*, 2009; Debache *et al.*, 2010; Pastor-Fernandez *et al.*, 2015). In addition, this vaccines could include specific markers allowing vaccinated and infected animals differentiation (Hemphill *et al.*, 2013). Unfortunately, the efficacy of the recombinant vaccines is low compared to live vaccines in mice and cattle (Monney and Hemphill, 2014; Hemphill *et al.*, 2016; Horcajo *et al.*, 2016).

5. Experimental models for toxoplasmosis and neosporosis

According to the National Research Council in USA, experimental models are a critical part in biomedical research and include animals, cell cultures as well as computational and mathematical simulations. Experimental models are important substitutes for studies that cannot be conducted in the target species and provide particular features or advantages that would otherwise be unavailable. In this way, many studies have inestimable worth in the clarification of major issues in different diseases and in the development of hypotheses to be tested in the target species (NRC, 1998).

Apart from that, the scientific world is increasingly aware with welfare and ethics in animal experimentation. In this context, research is based on the three R´s principle. This principle was stated at the beginning of the 1960s by two English biologists, Russel and Burch, in their book "The Principle of Humane Experimental Technique" (Russell *et al.*, 1959). The three R´s refer to replacement, reduction and refinement. Replacement alternatives refer to methods to avoid or substitute the use of animals. Whenever possible, *in vivo* procedures should be replaced by alternative methods that do not use animals, such as mathematical models or biological systems *in vitro*. Reduction means any strategy which results in the use of the lowest number of animals that allow to provide scientifically valid results, as well as maximization of the resulting information in each animal and thus, avoid or limit a later use of animals. The improvement of the experimental designs and the selection of the most appropriate model contribute to compliance of this principle. Finally, refinement alternatives include all the procedures to minimize or avoid suffering, as well as all the enrichment methods to ensure animal welfare.

In toxoplasmosis and neosporosis, experimental models are essential to increase knowledge in these diseases. Several *in vitro* models have been developed to study essential processes in the lytic cycle of *T. gondii* and *N. caninum* while *in vivo* models represent the best option to carry out therapeutic or vaccine trials, as well as to study pathogenesis, immune responses and outcomes of infection.

5.1. *In vitro* models

In vitro models have allowed to study mainly the infection of the tachyzoites in the host cell, simulating the dissemination of the parasite during the acute phase of the disease. *In vitro* cultivation of *T. gondii* and *N. caninum* makes possible execution of a large number of valuable experiments for the knowledge of the ultrastructural features of the parasites as well as the mechanisms of cell adhesion and invasion (Hemphill *et al.*, 2004; Lebrun *et al.*, 2014). Likewise, molecular basis of the lytic cycle and the host-parasite interactions have been studied, deepening on the knowledge of parasite mechanisms that module host cell response (Hemphill *et al.*, 2006).

Although *in vitro* models are not a solid alternative compared to the use of animal models, they provide an initial approach, optimizing the use of animals. *In vitro* models are used to characterize isolates, to carry out tachyzoite to bradyzoite conversion and to evaluate drug and vaccine candidates (Müller and Hemphill, 2013):

- **Characterization of isolates**: Among the parameters assessed, those related with invasion and proliferation rates are emphasized. Several comparative studies observed differences on these parameters between *T. gondii* isolates as well as *N. caninum* isolates. Regarding *T. gondii* isolates, the correlation between *T. gondii* virulence and growth rate has been extensively described (Kaufman *et al.*, 1958; Kaufman *et al.*, 1959). Thus, several studies have noted that type I strains (RH) grow faster than type II (ME49) or III strains (VEG) (Radke *et al.*, 2001). Likewise, *in vitro* differential phenotypic characteristics have been observed among type II *T. gondii* strains (Brenier-Pinchart *et al.*, 2010). Concerning *N. caninum* isolates, comparative studies have been carried out between Spanish bovine isolates (Nc-Spain 1H, 2H, 3H, 4H, 5H, 6, 7, 8, 9 y 10) and canine isolates (Nc-Ger2, Ger3, Ger6, Nc-6Arg y Nc-Bahia), using the Nc-Liverpool isolates as reference in both studies (Regidor-Cerrillo *et al.*, 2011; Dellarupe *et al.*, 2014b). Differences on the invasion and proliferation rates were observed, with isolates Nc-Spain4H, 5H, 6, 7, 10, Nc-Ger3, Nc-Bahia y Nc-Liverpool showing high virulence (higher invasion and lower time needed for replication). On the other hand, Nc-Spain 1H, 2H, 3H, 8, Nc-Ger2 y Ger6 showed a lower virulence (Regidor-Cerrillo *et al.*, 2011; Dellarupe *et al.*, 2014b).

- **Tachyzoite to bradyzoite conversion**: The development of methods for the tachyzoite to bradyzoite conversion *in vitro* provides an excellent tool to study antigen and protein composition, effect of prospective drugs and biology of both stages of the parasites (Lindsay *et al.*, 1993; Risco-Castillo *et al.*, 2007; Bougdour *et al.*, 2009; Marugán-Hernández *et al.*, 2010; Risco-Castillo *et al.*, 2011). The conversion to the latency stage has been associated with diverse responses to stress, and different methods to induce *in vitro* conversion has been evaluated. *N. caninum* tissue cysts have been more difficult to obtain using *in vitro* culture. Protocols developed for *T. gondii*, have yielded only few parasites undergoing stage conversion, showing that the efficiency of the differentiation process *in vitro* is rather low compared to *T. gondii* (Weiss *et al.*, 1999; Vonlaufen *et al.*, 2002). For tachyzoite to bradyzoite conversion *in vitro* in *N. caninum* the most effective method is adding to cell culture a nitric oxide donor, such as sodium nitroprusside, an apoptosis inducer (Vonlaufen *et al.*, 2002; Risco-Castillo *et al.*, 2004). For *T. gondii*, one of the earliest and most effective methods to induce tachyzoite to bradyzoite conversion *in vitro* is culturing infected cells in alkaline culture pH of 8–8.2 for three to four days (Skariah *et al.*, 2010).

- **Evaluation of drug and vaccine candidates**: *In vitro* models are gaining importance in recent years due to the usefulness in the evaluation of drugs against *N. caninum* and *T. gondii* (Doggett *et al.*, 2012; Mazuz *et al.*, 2012; Barna *et al.*, 2013; Ojo *et al.*, 2014; Winzer *et al.*, 2015; Müller *et al.*, 2017c), as well as vaccine candidates (Cho *et al.*, 2005; Staska *et al.*, 2005; Niedelman *et al.*, 2012; Alaeddine *et al.*, 2013). These *in vitro* models allow the evaluation of the impact of the compounds

on the parasites, and alterations on the parasites as well as modifications on the lytic cycle such as inhibition or slowdown in invasion, replication or egression can be assessed. Altogether, results provided using *in vitro* models allow an initial screening of the efficacy of the compounds, and therefore a selection of the best candidates to be tested in animal models.

• **Obtention of antigens**: *In vitro* culture has been also employed to obtain tachyzoites used as a source of antigen for diagnostic techniques and vaccine formulations (Alvarez-Garcia *et al.*, 2003; Ismael *et al.*, 2003; Aguado-Martínez *et al.*, 2008). For vaccine formulations, it is highlighted the use of tachyzoites for extraction of antigens and the expression of certain proteins in vectors for their purification and characterization (Garcia *et al.*, 2014; Monney and Hemphill, 2014).

5.2. Animal models of toxoplasmosis and neosporosis

Animal models are useful when, due to the characteristics of the target species or the objective of the study, there is no availability, their use is no practical and ethical, and is economically unviable or is not feasible to acquire and maintain a suitable number of animals of these species. Therefore, an animal model is a live organism in which it is possible to study a relevant fact that is similar to the target species (Wall and Shani, 2008). The choice of the animal model should be based on the suitability as analogous to the target species, the transferability of the information, uniformity, knowledge of the biological properties, generalization of the results, easy handling and ethical implications. However, these criteria with sense from a theorical point of view, not always can be considered in practice, due to punctual restrictions of each model.

Obviously the ideal model to study neosporosis is the bovine model, since it is the main target species, from both the veterinary and economic point of view, however, mice and ovine models are widely used. Regarding toxoplasmosis, due to the wide range of intermediate hosts, there are a large number of animal models in different species, and although the mice models are the most used due to the ease of handling, also ovine models have been established to study the disease. The main advantages of the use of mice as an experimental model are the small size, ease of handling, low cost, accessibility to many immunological reagents and availability of genetically modified animals. All these parameters together with the short period of gestation and the high number of pups facilitate greatly the generation of results in a short period of time. Therefore, mice models are useful for the characterization of isolates and study the pathogenesis of the diseases as well as for testing vaccine and drug candidates. Nevertheless, the choice an animal model will depend on the aim proposed, with each of them showing inherent pros and cons mainly related to the physiological characteristics of the animal species. Regardless the natural susceptibility of each species, and since the main impact of toxoplasmosis and neosporosis is the abortion, the physiological differences related to duration of gestation and, mainly, the type of placentation, are particularly relevant, since can play a critical role in the pathogenesis of toxoplasmosis and neosporosis.

In a schematic way, the placenta is composed by the maternal side or uterine lining, more or less modified, and the foetal side, with villi. The foetal elements of the placental barrier (trophoblast, connective tissue and foetal endothelium) are known as chorion. In accordance with morphological classification proposed by Strahl (Carter and Mess, 2010), ruminant placenta is multiple or cotyledonary, in which grouped villi are formed in small areas of the chorion. These correspond to elevations of the uterine lining called caruncles. The junction of the cotyledon (foetal side) and caruncle (maternal side) constitutes the placentome. The morphology of these placentomes is slightly different in ruminants (Figure 5). While in cow is convex, in sheep is concave and in goat is like a flat plateau. In rodents (as well as primates and humans), the placenta is discoidal, being the villi

grouped in a round or oval area. On the other hand, following the histologic classification proposed by Grosser based on extend of the destruction of maternal and foetal elements of the placenta (Grosser, 1927), the different species can be classified. Thus, bovine placenta is epitheliochorial since there is no destruction of maternal tissues, contacting the uterine epithelium with the chorion. The placenta in sheep and goats, despite being very similar to cattle, presents some differences. In this case, there is some areas with a syndesmochorial relation, which means a partial destruction of the uterine epithelium, attaching to trophoblast. Nevertheless, these areas present poor vascular supply, so from a functional point of view, is of low significance. Regarding mice, the placenta is hemochorial, with an extensive interaction of the foetuses with the maternal tissues, being in close contact the chorion and the maternal blood (Figura 5), therefore with a destruction of the vascular endothelium in the dam (Bainbridge, 2000).

Figure 5 – Morphology and structure of the placenta in ruminants and rodents

5.3. Animal models of toxoplasmosis

Since there are many hosts of *T. gondii*, a large number of animal models of toxoplasmosis have been established in very different species and with different purposes (Dubey, 2010). Thus, after the discovery of the parasite, the first isolation was conducted in mice (Sabin and Olitsky, 1937), although throughout its history, experimental infections have been also carried out in cat, the definitive host (Frenkel *et al.*, 1970).

In general, the purpose of studying *T. gondii* infection using experimental animal models is to obtain a better understanding of the disease in humans. The exception is in veterinary research, where it is possible to study experimental toxoplasmosis in the host of interest, e.g. sheep, goats or pigs. In this case the results are directly relevant to the disease in these animal species and are also of value in human toxoplasmosis.

T. gondii is capable of infecting all warm blooded animals; however, the consequences of infection are very variable between different species. Much of the work has been done using experimentally infected mice. While there are many advantages in using this experimental model, care should be taken in extrapolating results from mice to other species. Mice are vulnerable to the consequences of infection with *T. gondii*, and their use to further our understanding of congenital toxoplasmosis may not be ideal, as foetal infection can occur in successive generations of mice (Beverley, 1959). This is not the case in rats or sheep; they are more resistant to the disease and therefore may provide a more relevant model for human congenital toxoplasmosis (Innes, 1997). However, the correlation between virulence of *T. gondii* isolates in mice and other hosts remain unknown.

Beside mice, other experimental infections have been carried out in laboratory animals, such as rats (Dubey and Frenkel, 1998), rabbits and hares (Gustafsson *et al.*, 1997), chickens (Dubey, 2010), pigs (Dubey, 2009a), as well as in non human primates (Furuta *et al.*, 2001), Australian marsupials (Bettiol *et al.*, 2000), horses (Tassi, 2007), and ruminants such as sheep (see section 5.3.2 in this chapter), goats (Obendorf *et al.*, 1990) and cattle (Dubey, 1983). Finally experimental infections have been carried out in cats attempting to elucidate aspects of the enteroepithelial stages (Frenkel *et al.*, 1970; Dubey, 2001; Dubey, 2006) and other issues (Davidson *et al.*, 1993; Dubey *et al.*, 1995).

5.3.1. Mice model

Mice can be infected naturally by *T. gondii*, and an alarming high rate of *T. gondii* infection has been reported in house mice (*Mus musculus*)(Marshall *et al.*, 2004; Hughes *et al.*, 2006; Murphy *et al.*, 2008; Yan *et al.*, 2014), although much lower rates (0-5%) are more general (Smith *et al.*, 1992; Smith and Frenkel, 1995; Kijlstra *et al.*, 2008; Meerburg *et al.*, 2012; Muradian *et al.*, 2012; Hůrková-Hofmannová *et al.*, 2014).

Cat and mice are global species and sympatric and it is thought that mice is an evolutionary significant host for *T. gondii* with importance in the transmission of the pathogen (Müller and Howard, 2016). Virulence of *T. gondii* strains in mice varies with the genetic background of the parasites. Some genotypes of *T. gondii* are lethal to all strains of mice regardless of the dose of parasites administered, whereas other genotypes are non-lethal with a low dose of inoculation and can readily establish chronic infection in mice (Dardé *et al.*, 1992; Sibley and Boothroyd, 1992; Howe and Sibley, 1995; Ajzenberg *et al.*, 2002; Khan *et al.*, 2009a; Khan *et al.*, 2009b; Shwab *et al.*, 2016). Therefore, virulence of *T. gondii* for its natural intermediate host, the mouse, appears paradoxical from an evolutionary standpoint because death of the mouse before encystment interrupts the parasite life cycle (Lilue *et al.*, 2013).

Many mice models of toxoplasmosis has been established depending on the purposes of the study. Thus, the most used mice models are those to evaluate virulence of *T. gondii* isolates in mice and congenital infection, as well as those to study ocular toxoplasmosis (Dukaczewska *et al.*, 2015) and cerebral infection (Reichard and Gross, 2007).

- **Model to evaluate virulence of *T. gondii* isolates in mice**

The house mouse (*Mus musculus*) has been used as the primary laboratory animal model for determining the virulence of *T. gondii* strains. Thus, virulence of *T. gondii* strains has been commonly defined by the mortality rate in laboratory mice. Type I and most South American isolates are highly virulent in mice whereas types II and III show a dose-dependent mortality. However, many factors can affect virulence measurements, including route of infection, life stage of the parasite, number of passages of the parasite in mice or cell culture, the mouse host line used, and dose.

- Life stage of the parasite:

T. gondii has a complex life cycle, and the specific stages of the parasite used for inoculation may lead to marked differences in the outcomes of virulence in mice. All three infective forms may be used to infect mice, but infection with different forms may have varied results in terms of virulence. Among the three infectious stages of the parasite, oocysts in general are more virulent (Dubey and Frenkel, 1973; Dubey *et al.*, 1981; Dubey, 2006).

- Number of passages of the parasite in mice or cell culture:

Changes in biological characteristics occur in *T. gondii* strains after passage in mice or cell culture (Jacobs and Melton, 1954; Frenkel *et al.*, 1976; Lindsay *et al.*, 1991; Villard *et al.*, 1997; Dubey *et al.*, 1999; Khan *et al.*, 2009b). Several isolates *T. gondii* isolates were found to have lost the capacity to produce oocysts in cats after several passages (30-40) in cell culture and mice (Frenkel *et al.*, 1976; Lindsay *et al.*, 1991; Dubey *et al.*, 1999). A variety of phenotypic changes among several RH-derived clonal lineages were also observed (Khan *et al.*, 2009b). Differences among these lineages included larger plaque formation, enhanced survival outside the cells, faster growth, and decreased differentiation. Enhanced virulence in mice for *T. gondii* strains maintained in cell culture for several passages has been reported previously (Frenkel and Ambroise-Thomas, 1996). Genetic variability among RH lineages in different laboratories has been reported (Howe and Sibley, 1994); however, the cause-effect relationship between genetic variations and phenotypes is unclear. Therefore, any comparison of phenotypes among *T. gondii* isolates should be conducted using the original or low passage stocks.

- Route of infection:

The three common routes in determining *T. gondii* virulence in mice include intraperitoneal (IP) injection, subcutaneous (SC) injection and per oral inoculation (Dubey and Frenkel, 1973; Johnson, 1984). The IP injection involves direct deposition of *T. gondii* parasites into the peritoneal cavity, whereas SC injection deposits the parasites below the dermis. In oral infections, parasites are deposited into the stomach of mice. Higher infectivity and pathogenicity in mice have been observed between IP and oral route of infections with *T. gondii* compared to SC injection (Dubey and Frenkel, 1973; Dubey *et al.*, 1981). However, one study showed that mice were highly sensitive to oral infection, but resistant to IP injection (Johnson, 1984). As evident from these observations, the route of infection has an important effect on *T. gondii* virulence in mice and thus a standardized inoculation method is necessary in order for direct comparisons between different studies.

- Mouse line:

A high degree of variation in susceptibility to *T. gondii* has been observed among different lines of laboratory mice (Araujo *et al.*, 1976; Johnson, 1984; McLeod *et al.*, 1989; Suzuki *et al.*, 1993; Dubey *et al.*, 2012). Outbred mice were considerably resistant to *T. gondii* infection (Araujo *et al.*, 1976). Therefore it is important select the appropriate mouse lines with similar resistance when making virulence comparisons.

- Method of preparing tachyzoites for infection:

Most studies on *T. gondii* virulence in laboratory mice have employed the tachyzoites due to its relative ease of preparation. The two most common ways of propagating tachyzoites include the use of mice and cell culture (Dubey, 2010). For the former, *T. gondii* bradyzoites, tachyzoites or sporozoites can be inoculated into the mice by IP injection. Within a week post infection, tachyzoites can be harvested from the peritoneal exudates in the abdominal cavities of mice (Dempster, 1984). *T. gondii* isolates that are highly virulent usually propagate well in mice and release a large amount of free tachyzoites within 3-7 days post infection. However, *T. gondii* strains of low virulence usually replicate slowly and free tachyzoites are difficult to obtain. To overcome this problem, it is advisable to administer 10 mg/ml dexamethasone phosphate in drinking water, which will suppress the murine immune response and facilitate tachyzoites production (Dubey, 2010). In laboratories with access to cell culture, *T. gondii* tachyzoites can be propagated and maintained readily. Tachyzoites can proliferate in almost any mammalian cell lines, with fibroblasts being the most commonly used

(Derouin *et al.*, 1987). Whether obtained from cell culture or mouse peritoneal exudate, tachyzoites will often be mixed with a large amount of host cells. Therefore, it is necessary to remove these cells and enrich tachyzoites. There are several methods to purify tachyzoites, including density gradients, sonication and trypsin digestion, differential centrifugation, hemolysin digestion, filtration through glass wool, cellulose columns or sintered glass, and polycarbonate filtration (Dempster, 1984). With the exception of sonication and trypsin digestion, all of these methods are capable of removing greater than 90% of the mouse leukocytes without altering parasite viability (Dempster, 1984). Recently, a comparison of tachyzoite purification by trypsin digestion, filtration by 3-μm polycarbonate membrane, filtration by cellulose columns, and separation by percoll solution showed a similar results (Wu *et al.*, 2012). Therefore, tachyzoites prepared by trypsin digestion should be avoided for virulence testing in mice.

- Dose:

Earlier studies of mortality in mice have shown that some *T. gondii* strains such as those belonging to the type I lineage and most South American isolates are highly virulent to mice and kill all infected mice within two weeks after infection regardless of inoculation dosage (Dardé *et al.*, 1992; Sibley and Boothroyd, 1992). For these acutely virulent *T. gondii* strains, a single viable parasite is lethal to mice, therefore the mortality is dose-independent. In contrast, the majority of *T. gondii* strains can readily establish chronic infection in mice if inoculated at low dosages, and mortality is dose-dependent, with median lethal doses (LD_{50}) that range from 10^2 to 10^5 (Dardé *et al.*, 1992; Sibley and Boothroyd, 1992; Howe *et al.*, 1996; Khan *et al.*, 2009a, b). Therefore, to accurately determine the virulence of *T. gondii* isolates, it is necessary to test a series of parasite concentrations ranging from low to high inoculation doses. Any single dose inoculation will not be able to appropriately assess the virulence of a particular *T. gondii* isolate.

Variability among these factors makes it difficult to compare results between different studies in different laboratories, so recently it has been established a standardized method to evaluate *T. gondii* virulence in mice (Saraf *et al.*, 2017).

- **Mice model of congenital infection**

Mice are used for model for congenital infection due to the ease of handling and the similarity between mouse and human placenta, since both have a hemochorial placenta (Loke, 1982; Darcy and Zenner, 1993). As indicated by numerous articles that have appeared during decades, mice have been widely used to study congenital toxoplasmosis (Beverley, 1959; Remington *et al.*, 1961; Roberts and Alexander, 1992; Roberts *et al.*, 1994) and a review has been accomplished (Vargas-Villavicencio *et al.*, 2016).

- Primoinfection during pregnancy:

Maternal infection of *T. gondii* at early and intermediate stages of pregnancy could result in severe congenital toxoplasmosis, including foetal death (Hay *et al.*, 1983; Graham *et al.*, 1984; Couper *et al.*, 2003; Lahmar *et al.*, 2010; Wang *et al.*, 2011; Müller *et al.*, 2017c) and spontaneous cognitive handicap in the offspring (Wang *et al.*, 2011), while the infection at the late stage of pregnancy usually results in subclinical toxoplasmosis in the offspring (Cabañas-Cortés *et al.*, 2009; Wang *et al.*, 2011).

- Recrudescence of the infection:

Vertical transmission seems to occur during chronic infection and through successive generations of mice (Beverley, 1959; Remington *et al.*, 1961). Thus, at first glance the mouse model may not be best suited to mimic the situation in humans. However, other findings prove that whether transmission to the foetus during a chronic infection of the mother occurs is largely dependent on the parasite strain as well as on the mouse strain used – for example, in mice that were latently infected with 11 different *T. gondii* strains, placental transmission succeed with only 6 strains (Werner *et al.*, 1977), and, indeed, chronically infected BALB/c mice do not allow vertical disease transmission at all (Roberts and Alexander, 1992). As a consequence, more recent investigations on congenital toxoplasmosis tend to use a BALB/c mouse model rather than models using other inbred or outbred mouse strains (Thouvenin *et al.*, 1997; Fux *et al.*, 2000; Elsaid *et al.*, 2001; Abou-Bacar *et al.*, 2004; Abou-Bacar *et al.*, 2004; Beghetto *et al.*, 2005).

5.3.2. Ovine model

T. gondii is a main cause of reproductive failure in flocks worldwide, therefore, numerous ovine models have been established in order to evaluate the efficacy of drug (Buxton *et al.*, 1988; Buxton *et al.*, 1993a; Buxton *et al.*, 1996) and vaccine candidates (Buxton *et al.*, 1991; Buxton *et al.*, 1993b). Oocysts are the most frequent used in experimental infections (Dubey, 2009b), and despite not being natural routes of infection, tachyzoites (Hartley, 1961; Jacobs and Hartley, 1964; Payne *et al.*, 1988; Moraes *et al.*, 2010) and cysts (Jacobs and Hartley, 1964; Beverley *et al.*, 1975; Buxton and Finlayson, 1986; Verhelst *et al.*, 2014) have been also used for challenge.

- **Non-pregnant sheep model**

Non-pregnant sheep models have been used to evaluate effect of oocyst dose on clinical signs and serology (McColgan *et al.*, 1988), to develop serological techniques (Payne *et al.*, 1988; Verma *et al.*, 1989; Tenter *et al.*, 1992), as well as to study parasitaemia (Dubey and Sharma, 1980), parasite distribution and immune responses during the acute phase of the infection (Verhelst *et al.*, 2014) and lesions (Benavides *et al.*, 2011). Likewise, the protective effect of a primary infection on a subsequent infection (Falcon and Freyre, 2009) and the efficacy of toltrazuril on the formation of cysts (Kul *et al.*, 2013) have been studied. On the other hand, presence of *T. gondii* in semen of experimentally infected rams have been demonstrated (Teale *et al.*, 1982; Aganga *et al.*, 1988; Lopes *et al.*, 2009).

- **Pregnant sheep model**

Sheep can be easily infected by feeding them sporulated *T. gondii* oocysts (Table 1). Although in some studies there is a generally delay of 4 weeks until the occurrence of the abortion (Munday and Dubey, 1986), several experimental studies have defined an unacknowledged clinical presentation of ovine toxoplasmosis, where abortions occur during the acute phase of infection, between day 7 and 14 post infection (Dubey, 2009b). Most of the experimental infections have been carried out at mid-pregnancy, however, in a study comparing infections at early, mid and late gestation, early abortions were found regardless of the stage of gestation when ewes were infected (Castaño *et al.*, 2016). By contrast, there was a clear influence of the gestational stage over those abortions or stillbirths that occurred after 14 dpi. This influence is denoted by the differences in the rate and day post infection in which abortions occur, histological lesions and parasite burden (Castaño *et al.*, 2016).

A range of variables, including isolate virulence and infective dose, have been proposed as key factors in the development of the early abortions (Benavides *et al.*, 2017). Although early abortions in sheep have been observed after infection with type I (GT-1 strain) (Dubey, 1984) and type II (M1,

M3, M4 and PRU) isolates (Trees *et al.*, 1989; Buxton *et al.*, 1996; Mévélec *et al.*, 2010; Castaño *et al.*, 2014), little is known about the influence of the isolate virulence on this clinical form of the disease. Concerning doses of infection, a large range, between 12000 (Buxton *et al.*, 1988) and 50 oocysts (Castaño *et al.*, 2016), has been evaluated in sheep at mid-pregnancy, however there are only three experimental infections in which intermediate-to-high oocyst doses were titrated (Buxton *et al.*, 1988; Trees *et al.*, 1989; Mévélec *et al.*, 2010). Most of the ewes fed 2000 oocysts or more aborted or produced dead foetuses, although 100% abortion were reported in a study after infection with 100 oocysts (Mévélec *et al.*, 2010) (Table 1).

Artificial insemination using semen contaminated with 6.5×10^4 and 4×10^7 *T. gondii* tachyzoites can also cause reproductive pathology with embryonic reabsorption in 80% and 100% of infected ewes (Moraes *et al.*, 2010). Primary infection of pregnant sheep has been also useful to evaluate presence and quantity of *T. gondii* in maternal and foetal tissues (Gutierrez *et al.*, 2010). Although the importance of endogenous transplacental transmission of *T. gondii* in naturally infected sheep remains controversial (see section 2.3 above in this chapter), after experimental challenge before pregnancy, congenital infection was common (dos Santos *et al.*, 2016).

Table 1 – Experimental toxoplasmosis in sheep primary induced orally with oocysts during pregnancy

Reference	Isolate	Dose[1]	Day of preg.[2]	Aborted sheep/dead foetuses[3]	Live lambs[4]
Beverley *et al.*, 1975	Beverley	10000	78-91	8/8 (28 dpi)	0/15
Dubey, 1984	GT-1	10000	102	3/8 (7-49 dpi) serial culling	NA (serial culling)
Munday and Dubey, 1986	GT-1	1500	42-98	12/19 (26-55 dpi)	7/21
Aganga *et al*., 1988	TS-1, 2 Nigerian isolates	10000	90	9/9 (10-30 dpi)	None
Buxton *et al*., 1988	M1	2000-12000	91-94	16/19 dead foetuses (10-48 dpi)	13/29
Trees *et al*., 1989	M1	2000-12000	91-94	11/20 (12-53 dpi)	NA
Buxton *et al.*, 1989	M1	2000	91-92	11/17 dead foetuses (40 dpi)	6/17
Fredriksson et al., 1990	M1	2000	91-92	9/13	NA
Buxton *et al.,* 1991	M3	2000	89-90	37/45 dead foetuses (8-41 dpi)	8/45
Kirkbride *et al.,* 1992	TS-1	100	55	14/17 (105-135 dpi)	3/31
Buxton *et al.*, 1993b	M3	2000	89-91	40/45 dead foetuses	5/45
Buxton et al., 1996	M3	200	90	7/29 (11-30 dpi)	22/58
Owen *et al*., 1998a	M1	2000	80-90	11/15 (10-13 dpi)	3/14
Owen *et al.,* 1998b	M1	1500	80-90	5/18	7/21
Mévelec *et al*., 2010	PRU	400	90	21/21 (early and late abortions)	0/21
		100		6/6 (early and late abortions)	0/10
Castaño *et al*., 2014	M4	2000	90	11/12 (7-11 dpi) serial culling	None
		500	120	7/12 (9-11 dpi) serial culling	None
Castaño *et al*., 2016	M4	50	40	3/9 (12-26 dpi) serial culling	NA
			90	5/9 (11-26 dpi) serial culling	NA
			120	6/9 (9-26 dpi) serial culling	NA

Modified from Dubey (2010)

(1) Number of oocysts.

(2) Days of pregnancy when challenge occurs (gestation in sheep lasts around 148 days).

(3) Fractions indicate the number of aborted sheep/dead foetuses in relation to the total number of sheep challenged or foetuses. In brackets is indicated the day or range in which abortions ocurr.

(4) Fractions indicate the number of live lambs in relation to the total number of lambs. (NA) No applicable.

5.4. Animal models of neosporosis

In the case of neosporosis, the advancement of animal models has been essential, allowing a great progress in its knowledge. In fact, shortly after the discovery of *N. caninum*, the first experimental models based on parasite inoculations in mice and rats (Lindsay and Dubey, 1989b; Lindsay and Dubey, 1990b) and sheep (Dubey and Lindsay, 1990) begin implementation. Later, deeper understanding on the pathogenesis was achieved through experimental infections in mice, cattle and sheep (Barr *et al.*, 1994; Lindsay *et al.*, 1995; McAllister *et al.*, 1996b).

Obviously the ideal model to study neosporosis is the bovine model, however, due to economical and logistical limitations, only a few institutions are able to carry out such experiments. Therefore, laboratory animals, mainly mice, have become a very attractive alternative for different researchers. (Cole *et al.*, 1995; Lindsay *et al.*, 1995; Long and Baszler, 1996; Collantes-Fernández *et al.*, 2006a; López-Pérez *et al.*, 2008). Beside mice, other laboratory animals have shown susceptibility to experimental *N. caninum* infection, such as gerbils (Dubey and Lindsay, 2000; Gondim *et al.*, 2001; Ramamoorthy *et al.*, 2005; Kang *et al.*, 2009), chickens and pigeons (Furuta *et al.*, 2007; Mineo *et al.*, 2008) or rats, the latter present some natural resistance and a immunosuppressive treatment is needed to generate infection (Lindsay and Dubey, 1990b). Rabbits have been mainly used to generate antibodies against the parasite, although these animals remain clinically healthy even with high infectious doses (Lindsay and Dubey, 1989a). Finally, despite not numerous, experimental infections have been carried out in dogs attempting to elucidate aspects of the enteroepithelial stages (McAllister *et al.*, 1998; Lindsay *et al.*, 1999; Cedillo *et al.*, 2008; Bandini *et al.*, 2011; Cavalcante *et al.*, 2012; Munhoz *et al.*, 2013). On the other hand, the use of other ruminants as an alternative model has the advantage of being very closely related phylogenetically with cattle. Within these ruminants, buffalos (Konrad *et al.*, 2012), goats (Lindsay *et al.*, 1995; Yin *et al.*, 2012; Porto *et al.*, 2016) and sheep have been used, being the latter the most frequently used (detailed above).

5.4.1. Mice model

Although there are some evidences that rodents can be naturally infected by *N. caninum*, since the parasite has been detected by PCR in mice and rats (Huang *et al.*, 2004; Ferroglio *et al.*, 2007; Jenkins *et al.*, 2007; Medina-Esparza *et al.*, 2013), their role in the biological cycle is unknown. Along with the low susceptibility of mice to neosporosis, the main disadvantage of this model is the marked physiological differences with cattle, the target host. In this sense, immune response and different gestation parameters (particularly length of gestation and placental structure) make results difficultly extrapolated directly to the targe host. However, mice models are the most used model at present.

Mice models are widely heterogeneous and the most used are the non-pregnant mice models or mice models of cerebral infection (Collantes-Fernández *et al.*, 2006a; Pereira García-Melo *et al.*, 2010) and the pregnant mice models or mice models of congenital infection (López-Pérez *et al.*, 2006; López-Pérez *et al.*, 2008; Arranz-Solis *et al.*, 2015a).

- **Non-pregnant mice model**:

In this model, and according to the intraorganic distribution of the parasite, kinetics of the immune response and presence and severity of clinical signs, the course of the infection can be separated into two phases.

- Acute phase: during this phase of infection the mice can start to show unespecific clinical signs such as inactivity, anorexia, apathy, ruffled coat or dyspnea in the more severe cases (Eperon *et al.*,

1999; Rettigner *et al.*, 2004a; Collantes-Fernández *et al.*, 2006a). Usually, except in immunodeficient mice, there is no mortality in this phase of infection.

- Chronic phase (cerebral model): in the second week pi, a progressive decrease in the number of tachyzoites in the lung is observed, as well as a higher load in the brain (Collantes-Fernández *et al.*, 2006a; Pereira García-Melo *et al.*, 2010). Mice can be asymptomatic or show nervous clinical signs such as weakness, paralysis of the hindlimb, rounded back, head tilt, ataxia and circular movements (Eperon *et al.*, 1999; Rettigner *et al.*, 2004a; Collantes-Fernández *et al.*, 2006a). These clinical signs get progressively worse causing death of the animal (Lindsay and Dubey, 1989b; Reichel and Ellis, 2009).

- **Pregnant mice model**:

The pregnant mice model has proven to be particularly useful to study congenital neosporosis, being important to evaluate the safety and the efficacy of vaccines and drugs. In fact, the use of the pregnant mice model as a proof of concept before the study in the target host is a more adequate alternative than the non-pregnant mice model, since the goal of the compounds is avoid vertical transmission of the parasite (Aguado-Martínez *et al.*, 2009; Marugán-Hernández *et al.*, 2011; Rojo-Montejo *et al.*, 2011; Monney *et al.*, 2012; Debache and Hemphill, 2013; Arranz-Solis *et al.*, 2015a). Also, in spite of the physiological differences between both species, most of the results from this model have shown a high degree of similarity with those from the bovine model.

To date, most of the models of congenital neosporosis simulate the exogenous transplacental transmission, with a primoinfection during pregnancy (Liddell *et al.*, 1999b; Quinn *et al.*, 2002; López-Pérez *et al.*, 2006; López-Pérez *et al.*, 2008; Arranz-Solis *et al.*, 2015a). Concerning recrudescence of the infection, the attempts to reproduce endogenous transplacental transmission have not been satisfactory (Jiménez-Ruiz *et al.*, 2013a, b).

5.4.2. Bovine model

Obviously, the main advantage of the use of the bovine model is that the results are directly applicable and transferable to the knowledge and control of neosporosis. Nevertheless, the requirements for the experimentation with these animals (high cost, extended time of the experiments, hard handling of the animals, appropriate facilities, etc.) make impossible their use in initial tests of drug and vaccine candidates. In addition, the analysis of different conditions in the same experiment is greatly hampered by the limited number of experimental animals. Thus, the bovine model, as target host from both the veterinary and economic point of view, represents de model of choice to study the outcomes of infection (Benavides *et al.*, 2014). This model is very useful to study important aspects of neosporosis, such as pathogenesis, host-parasite interaction, local and systemic immune responses. Furthermore, bovine model is essential for the final evaluation of prospective vaccine and drug candidates, as well as new diagnostic tools. In any case, different bovine models, both non-pregnant and pregnant, have been established (Benavides *et al.*, 2014).

- **Non-pregnant bovine model**:

Most of the experiments conducted in non-pregnant bovine models have been carried out in order to study the *N. caninum* life cycle and the postnatal modes of transmission. In this sense, it was proven the oral mode of transmission between dog and cattle (Gondim *et al.*, 2002).

- **Pregnant bovine model**:

Although endogenous transplacental transmission has been considered the most common mode of transmission and maintenance of neosporosis in the herd (Dubey *et al.*, 2007), to date, the reproduction in bovine experimental models has not been achieved (McCann *et al.*, 2007; Benavides *et al.*, 2014). Thus, most of the pregnant models currently available are models of primoinfection to evaluate exogenous transplacental transmission. In the bovine model of primoinfection, the most important parameters to be evaluated are abortion and vertical transmission.

5.4.3. Ovine model

Natural *N. caninum* infection in sheep was first described in a congenitally infected lamb (Dubey and Lindsay, 1990). Later, ovine neosporosis has been described in several cases worldwide (Kobayashi *et al.*, 2001; Koyama *et al.*, 2001; Hassig *et al.*, 2003; Moore, 2005; West *et al.*, 2006; Howe *et al.*, 2012; Moreno *et al.*, 2012; González-Warleta *et al.*, 2014). Nevertheless, traditionally *T. gondii* has been considered the main parasite causing abortion in sheep, with *N. caninum* being much less epidemiologically, clinically and economically relevant compared to cattle (Buxton, 1998; Givens and Marley, 2008; Dubey and Schares, 2011). However, recent studies have demonstrated that *N. caninum* could be an important cause of abortion in small ruminants (Moreno *et al.*, 2012), and even of reproductive losses in many flocks (West *et al.*, 2006; González-Warleta *et al.*, 2014; González-Warleta et al., submitted). Several studies in different countries have shown a highly variable *N. caninum* seroprevalence in sheep. Thus, for example, it has been described a global seroprevalence in the world ranging from 2% to 14% (Hassig *et al.*, 2003; Moore, 2005; Howe *et al.*, 2012). However, in Jordan a seroprevalence rate of 92% has been described (Abo-Shehada and Abu-Halaweh, 2010), while in Spain a seroprevalence rate of 5% was reported (Díaz *et al.*, 2014). On the other hand, *N. caninum* detection in aborted foetuses has been examined in several studies. For example, detection percentage in aborted foetuses was 20% in Switzerland (Hassig *et al.*, 2003), 13% in New Zealand (Howe *et al.*, 2012) and 7% in Spain (Moreno *et al.*, 2012), being the detection percentage in the latter similar for *T. gondii.*

Besides being a natural *N. caninum* host, ovine model has some advantages over bovine model, such as shorter gestation period and easy handling. Furthermore, this model allows the use of a larger number of animals. Likewise, ovine placenta is very similar compared to cattle. Thereby, ovine model is a good alternative to the use of expensive bovine model and mice model. Nonetheless, despite being closely related phylogenetically, there are evidences that *N. caninum* infections in cattle and sheep are not exactly the same (Benavides *et al.*, 2014). However, the results from the ovine model are more extrapolable to bovine model than those from the murine model. Thus, ovine model results particularly helpful to test vaccines and drugs. Since the discovery of *N. caninum*, several experimental infections in sheep have been carried out, but less compared to bovine and mice (Benavides *et al.*, 2014). Most of them are focused on the study of pathogenesis and immune response of *N. caninum* during pregnancy.

- **Non-pregnant sheep model**:

Just like bovine models, most of the studies in non-pregnant sheep aimed to deep the knowledge of the *N. caninum* life cycle (Benavides *et al.*, 2014). Thus, for example, horizontal transmission has been demonstrated in sheep after ingestion of oocysts shed by dogs as well as in dogs after feeding with tissues from these sheep (Schares *et al.*, 2001). Likewise, sheep model has been used to study venereal mode of transmission. In a *N. caninum* experimental infection of rams, it was demonstrated the dose-dependent presence of tachyzoites in the semen after infection, albeit it was no able to infect female sheep at mating, similarly to findings in cattle (Syed-Hussain *et al.*, 2013).

- **Pregnant sheep model**:

Experimental infections in pregnant sheep have shown a similar pattern than bovine neosporosis, with abortions, healthy but congenitally infected lambs or lambs showing weakness and neurological signs depending on the stage of gestation when infection occurs. Most of the experimental infections in pregnant sheep have been carried out at mid-gestation, while those in early and late gestation are scarce (Table 2). In these studies it was observed that infections early in gestation usually result in abortion (Buxton *et al.*, 1998; Arranz-Solis *et al.*, 2015b), while infections in the last term of gestation cause the birth of healthy but congenitally infected lambs (McAllister *et al.*, 1996b; Arranz-Solis *et al.*, 2015b; Syed-Hussain *et al.*, 2015a). However, the likelihood of abortion after infection at mid-pregnancy in sheep is much higher compared to cattle (Dubey and Lindsay, 1990; McAllister *et al.*, 1996b; Buxton *et al.*, 1998; Buxton *et al.*, 2001; Innes *et al.*, 2001a; Weston *et al.*, 2009; Arranz-Solis *et al.*, 2015b). Nevertheless, in sheep it has been also described the birth of congenitally infected lambs after infection at mid-pregnancy (McAllister *et al.*, 1996b; Buxton *et al.*, 1998; O'Handley *et al.*, 2003).

Apart from the stage of gestation, several authors revealed the importance of the infection dose, with higher percentage of abortions after infection with high doses (Dubey and Lindsay, 1990; Buxton *et al.*, 2001; Innes *et al.*, 2001a; Weston *et al.*, 2009), while the infection with low doses reduces abortion or not appear despite causing infection (Weston *et al.*, 2009).

Finally, there is no a model of recrudescence in sheep so far. Several studies have evidenced recrudescence of the infection in natural conditions (González-Warleta et al., submitted) or even experimentally (Jolley *et al.*, 1999). Despite this, other studies did not detect abortions in sheep experimentally infected 3 and 8 weeks before gestation (Buxton *et al.*, 2001; Syed-Hussain *et al.*, 2015b). However, the latter observed vertical transmission since parasite was detected in more than the 50% of healthy lambs (Syed-Hussain *et al.*, 2015b).

Table 2 – *N. caninum* experimental infections in sheep during pregnancy

Reference	Isolate	Dose[1]	Route[2]	Day of preg.[3]	Aborted sheep[4]	Live lambs[5]
Dubey & Lindsay, 1990	Nc-1	$1,5\times10^7$	IV	90	1/1 - 25 dpi	0/2
			IM		1/1 - 26 dpi	0/2
McAllister *et al*., 1996b	Nc-2+ Nc-Liverpool	$1,7\times10^5$	IV	65	6/6 - 53 dpi (36-69)	0/9
		$1,7\times10^6$			6/6 - 45 dpi (41-51)	0/10
		$1,7\times10^5$		90	4/6 - 49 dpi (41-59)	4/9 (2 weak and 2 healthy lambs)
		$1,7\times10^6$			4/6 - 47 dpi (36-61)	3/8 (2 weak and 1 healthy lambs)
		$1,7\times10^5$		120	0/6	7/7
		$1,7\times10^6$			0/6	6/6
Buxton *et al*., 1997	Nc-Liverpool	10^6	SC	90	0/12 (serial culling)	NA (serial culling)
Buxton *et al*., 1998	Nc-1	10^6	SC	45	8/8 – (42-62 dpi)	0/10
				65	7/8 – (35-78 dpi)	1/11
				90	6/8 – (35-44 dpi)	4/11
Innes *et al.*, 2001a	Nc-1	10^7	SC	90	12/12 – 41 dpi	0/14
Buxton *et al*., 2001	Nc-1	10^7	SC	90	12/12 - 41 dpi (31-59)	0/14
O'Handley *et al*., 2003	Nc-Illinois	5×10^6	SC	68-108	0/20	35/38 (3 stillborn lambs, 28 healthy lambs, 7 lambs dead during the 1st week)
Jenkins *et al*., 2004b	Nc-Illinois	5×10^6	SC	45-75	5/9	6
Weston *et al*., 2009	Nc-NZ1 + Nc-NZ2 + Nc-NZ3	50	IV	73-90	0/10	10/10
		5000			5/10 – 49 dpi (36-69)	5/10 (2 premature and 3 healthy lambs)
		10^6			10/10 - 45 dpi (37-54)	0/10
		10^8			10/10 - 38 dpi (26-56)	0/10
Syed-Hussain *et al.*, 2015b	Nc-NZ1	10^7	IV	120	0/12	17/17 (7 lambs dead during the 1st week)
Arranz-Solis *et al*., 2015b	Nc-Spain7	10^6	IV	40	6/6 – (19-21 dpi)	0/11
				90	7/7 – (34-48 dpi)	0/12
				120	0/7	9/9 (3 weak lambs)

(1) Number of tachyzoites inoculated.
(2) (IV) Intravenous; (IM) intramuscular; (SC) subcutaneous
(3) Days of pregnancy when challenge occurs (gestation in sheep lasts around 148 days).
(4) Fractions indicate the number of aborted sheep in relation to the total number of sheep challenged. Days post-infection (dpi) in which abortions occur. In brackets is indicated the range.
(5) Fractions indicate the number of live lambs in relation to the total number of lambs.. (NA) No applicable.

CAPÍTULO III

JUSTIFICACIÓN Y OBJETIVOS

JUSTIFICATION AND OBJECTIVES

“La religión es la cultura de la fe, la ciencia es la cultura de la duda”

Richard Feynman (1918-1988)

T. gondii and *N. caninum* are apicomplexan parasites considered to be one of the main infectious causes of abortion in sheep and cattle, respectively. Toxoplasmosis and neosporosis are generally latent and asymptomatic in non-pregnant sheep/cattle, although the consequences of infection in a pregnant sheep/cow can be abortion, birth of weak lambs/calves or birth of clinically healthy but persistently infected lambs/calves (Innes *et al.*, 2002). *T. gondii* and *N. caninum* show a high prevalence rate and important economic losses in ovine and cattle population, respectively. In sheep, primoinfection with *T. gondii* during pregnancy triggered reproductive failure, while the recrudescence of a latent infection is rare and seems to play a minor role in transmission of *T. gondii* in sheep (Trees and Williams, 2005). However, reproductive failure in cattle due to *N. caninum* infection can be found after infection during pregnancy or recrudescence of a chronic infection (Dubey and Schares, 2011). For the control of toxoplasmosis in sheep there is a commercial attenuated live vaccine available, which partially reduced abortions (Buxton and Innes, 1995), while no vaccine is available in the market for the prevention of neosporosis in cattle. Concerning treatment, to date, no drug is available for the treatment of toxoplasmosis and neosporosis in ruminants. Therefore, the improvement and development of control measures is of paramount importance. Indeed, the efforts of several research groups are nowadays focused on the search for effective drugs and vaccines against ovine toxoplasmosis and bovine neosporosis.

In this sense, the employment of *in vitro* models has been a great help to the increase on the knowledge of ultrastructural features of the parasite and to gain deeper insight into the processes of the tachyzoite lytic cycle (Hemphill *et al.*, 2004; El Hajj *et al.*, 2007). This understanding has allowed the use of *in vitro* models for various assays and assessments. Among these, the obtainment and characterization of isolates, the evaluation of putative drugs and the tachyzoite-bradyzoite conversion studies stand out. Although *in vitro* assays does not pose a sound alternative to the employment of animal models, they offer an initial approximation in many variables of interest, thus reducing and optimising the usage of experimentation with animals (3R´s rule). However, the use of animal models is essential for the study of several aspects of the disease, which cannot be analysed by *in vitro* means. In fact, *in vivo* models have increased and deepened the knowledge of toxoplasmosis and neosporosis and have allowed to test potential drugs and vaccine candidates.

Mice is often used as the preferred animal model to determine the virulence of *T. gondii* (Saraf *et al.*, 2017). Likewise, to evaluate congenital infection, several mice models are available (Vargas-Villavicencio *et al.*, 2016), since the use of laboratory mice is of advantage in terms of low cost, easy handling, short gestation period and litter size. However, structure of the placenta, reproductive physiology and immune responses greatly differ between rodents and ruminants, which clearly could influence the passing of *T. gondii* trough the maternofetal interface and the modulation of the host immune responses during pregnancy. In sheep, most of the experimental infections during pregnancy have been carried out using the M1, M3 and M4 *T. gondii* type II isolates (Dubey, 2009b; Castaño *et al.*, 2014) and the virulence in mice of these strains has not been studied in depth. Therefore, there is no information on how *T. gondii* virulence in mice compare to the outcome of experimental infection in pregnant sheep.

In neosporosis, the interest of pregnant sheep models has arisen in the last years as a valid alternative to the costly bovine model and to the poorly comparable murine model. In this sense, sheep provide several advantages over cattle as an experimental animal model, such as number of animals, ease of handling, length of the experiment and cost. Moreover, sheep are, likewise other ruminants, a natural intermediate host of *N. caninum* (Dubey *et al.*, 2007). In fact, recent studies in small ruminants suggest that neosporosis may be a more important cause of reproductive disorders than it has been traditionally considered, at least in certain scenarios. Recent evidence has

accumulated, which points towards *N. caninum* as a cause of natural abortion and reproductive failure in sheep and goats (West et al., 2006; Moreno et al., 2012; González-Warleta et al., 2014; Benavides et al., 2014; González-Warleta et al., submitted). The pathogenesis of ovine neosporosis is poorly understood, infection during mid-pregnancy in sheep results in severe clinical outcome, since most of the animals abort or, less frequently, produce weak lambs (McAllister *et al.*, 1996b; Arranz-Solis *et al.*, 2015b). In pregnant sheep, infective doses of 10^7– 10^8 tachyzoites result in a high percentage of abortions (Dubey and Lindsay, 1990; Buxton *et al.*, 2001; Innes *et al.*, 2001a; Weston *et al.*, 2009) and there is only one study comparing different infective doses (Weston *et al.*, 2009). In the latter, the results are difficult to compare since a mixture of isolates was employed (Weston *et al.*, 2009). Likewise, although the subcutaneous inoculation closely mimics a natural primary infection as the parasite is "processed" through a draining lymph node before circulating in the blood (Dubey *et al.*, 2006), nowadays, there are no studies comparing the outcome of *N. caninum* experimental infection using different routes of inoculation in pregnant sheep.

To date, pregnant ruminant models have not been used for assessments of drug efficacy against *N. caninum* infection and vertical transmission. In toxoplasmosis, drugs evaluated in pregnant sheep (monensin, folate inhibitors and decoquinate) showed protection against abortion in 10-40% of infected ewes, however there is limited or no protection against vertical transmission. Therefore, there is lack of effective drugs for the treatment of congenital toxoplasmosis and neosporosis in ruminants. Highly promising drugs classes have been tested against *T. gondii* and *N. caninum in vitro* and in small animal models, such as thiazolides, diamidines, artemisinins, naphthoquinones, anticancer agents, endochin-like quinolones and calcium-dependent protein kinase inhibitors (Sánchez-Sánchez et al., accepted for publication). Calcium dependent protein kinase 1 (CDPK1) represents a promising drug target, as CDPK1 is encoded by the apicoplast DNA, and is thus absent from mammalian hosts (Lourido *et al.*, 2010; Murphy *et al.*, 2010; Ojo *et al.*, 2010) and it is conserved among apicomplexan parasites (Keyloun *et al.*, 2014). CDPK1 activity is essential for microneme secretion, host cell invasion, and egress of *T. gondii* (Kieschnick *et al.*, 2001; Lourido *et al.*, 2010) and can be effectively targeted by a class of ATP-competitive compounds, collectively named bumped kinase inhibitors (BKIs). The compound BKI-1294 was effective *in vitro* against *T. gondii* and *N. caninum* and showed highly protection against vertical transmission in mice models of congenital toxoplasmosis and neosporosis (Winzer *et al.*, 2015; Müller *et al.*, 2017c). Likewise, the compound BKI-1553, with an improved bioavailability compared to BKI-1294 (Vidadala *et al.*, 2016) was also effective *in vitro* against *N. caninum* and *in vivo* in a pregnant mice model of neosporosis (Müller *et al.*, 2017b).

In this scenario, the general aim of the present Doctoral Thesis was to standardize pregnant mice and sheep models of *T. gondii* infection and compare the virulence of *T. gondii* type II isolates between mice and sheep, standardize a pregnant sheep model for the study of *N. caninum* infection, and evaluate the safety and efficacy against *T. gondii* and *N. caninum* infections of BKI compounds in pregnant sheep.

To this end, in the first place, the *in vitro* phenotype and *in vivo* behavior in mice and sheep of two type II *T. gondii* isolates were determined. Secondly, a dose titration of *N. caninum* and the evaluation of the intravenous and subcutaneous routes of administration in pregnant sheep were carried out in order to assess the impact on this model. Finally, the safety and efficacy against *T. gondii* and *N. caninum* infections of BKI-1294 and BKI-1553 compounds, respectively were evaluated.

- **Objective 1. Standardization of pregnant mice and sheep models of *T. gondii* infection and comparison of the virulence of *T. gondii* type II isolates in mice and sheep.**

This objective included the evaluation of the effects of infection in mice and sheep of a recently obtained type II *T. gondii* isolate (TgShSp1) and the reference type II *T. gondii* isolate (TgME49) with the aim of determine if virulence in mice of *T. gondii* type II isolates could be a good indicator to predict the outcome of experimental infection in pregnant sheep.

- **Objective 2. Standardization of the pregnant sheep model of *N. caninum* infection by a dose-titration assay and evaluation of different routes of administration of the virulent Nc-Spain7 isolate.**

This study was conducted to evaluate the effects of various infectious doses and routes of administration of a virulent *N. caninum* isolate (Nc-Spain7) in pregnant sheep, with the aim of optimising the challenge dose and route of administration, and thus to contribute to the refinement and standardization of the pregnant sheep model for its use in further studies.

- **Objective 3. Evaluation of the safety and efficacy against *T. gondii* and *N. caninum* of BKI compounds in pregnant sheep.**

These studies were carried out to assess the safety of BKI-1294 orally administered and BKI-1553 subcutaneously administered in pregnant sheep and their efficacy against abortion and vertical transmission after experimental infection with *T. gondii* and *N. caninum*, respectively, with the aim of find therapeutic tools for the control of toxoplasmosis and neosporosis in ruminants.

CAPÍTULO IV

RESULTADOS (PUBLICACIONES)

RESULTS (PUBLICATIONS)

“Para mí, nunca ha habido una mayor fuente de honores terrenales o distinción mayor que la conexión con los avances de la ciencia”

Isaac Newton (1642-1727)

LIST OF PUBLICATIONS

Objective 1: Standardization of pregnant mice and sheep models of *T. gondii* infection and comparison of the congenital infection of *T. gondii* type II isolates in mice and sheep.

Sánchez-Sánchez R, Ferre I, Regidor-Cerrillo J, Gutiérrez-Expósito D, Ferrer LM, Arteche-Villasol N, Moreno-Gonzalo J, Müller J, Aguado-Martínez A, Pérez V, Hemphill A, Ortega-Mora LM, Benavides J. Virulence in mice of a *Toxoplasma gondii* type II isolate does not correlate with the outcome of experimental infection in pregnant sheep. Submitted to "Frontiers in Cellular and Infection Microbiology", research topic "*Toxoplasma gondii* Host Interactions: A Story of Immune Attack and Parasite Counterattack" on day 18th September 2018.

Objective 2: Standardization of the pregnant sheep model of *N. caninum* infection by a dose-titration assay and evaluation of different routes of administration of the virulent Nc-Spain7 isolate.

Sánchez‐Sánchez R, Ferre I, Re M, Regidor‐Cerrillo J, Blanco‐Murcia J, Ferrer LM, Navarro T, Pizarro Díaz M, González‐Huecas M, Tabanera E, Benavides J, Ortega‐Mora LM. Influence of dose and route of administration on the outcome of infection with the virulent *Neospora caninum* isolate Nc‐Spain7 in pregnant sheep at mid‐gestation. Published in Veterinary Research (8th May 2018) 49:42. DOI: 10.1186/s13567-018-0539-5.

Objective 3: Evaluation of the safety and efficacy against *T. gondii* and *N. caninum* of BKI compounds in pregnant sheep.

Sánchez-Sánchez R, Ferre I, Re M, Ferrer LM, Regidor-Cerrillo J, Pizarro Díaz M, González-Huecas M, Tabanera E, Benavides J, Hemphill A, Hulverson MA, Barrett LK, Choi R, Whitman GR, Ojo KK, Van Voorhis WC, Ortega-Mora LM. Bumped kinase inhibitor BKI-1294 is safe and produces high rates of protection against abortion and vertical transmission in sheep experimentally infected with *Toxoplasma gondii* during pregnancy. To be submitted to Journal of Antimicrobial Chemotherapy

Sánchez-Sánchez R, Ferre I, Re M, Vázquez P, Ferrer LM, Blanco-Murcia J, Regidor-Cerrillo J, Pizarro Díaz M, González-Huecas M, Tabanera E, García-Lunar P, Benavides J, Castaño P, Hemphill A, Hulverson MA, Whitman GR, Rivas KL, Choi R, Ojo KK, Barrett LK, Van Voorhis WC, Ortega-Mora LM. Safety and efficacy of the bumped kinase inhibitor BKI-1553 in pregnant sheep experimentally infected with *Neospora caninum* tachyzoites. Published in International Journal for Parasitology: Drugs and Drug Resistance (2nd March 2018). 8 (2018) 112‐124. DOI: 10.1016/j.ijpddr.2018.02.003.

Appendix I: Literature review.

Sánchez-Sánchez R, Vázquez P, Ferre I, Ortega-Mora LM. Treatment of toxoplasmosis and neosporosis in farm ruminants: state of knowledge and future trends**.** Accepted for publication in Current Topics in Medicinal Chemistry (21th March 2018). It will be published in Volume 18.

Objetivo 1

Estandarización de los modelos murino y ovino gestantes de infección por *T. gondii* y comparación de la virulencia de aislados tipo II de *T. gondii* entre ratón y oveja

Toxoplasma gondii es un párasito apicomplejo que infecta a casi todos los animales de sangre caliente. Se conoce poco acerca de como la virulencia del parásito en ratón se extrapola a otro hospedadores relevantes. En el presente estudio, se comparan el fenotipo *in vitro* y el comportamiento *in vivo* en ratón y en oveja de un aislado tipo II de *T. gondii* (TgShSp1) y del aislado de tipo II de *T. gondii* de referencia (TgME49). Los resultados de los ensayos *in vitro* y de la inoculación intraperitoneal de taquizoitos en ratones indicaron una virulencia incrementada del aislado de laboratorio TgME49 en comparación con el aislado recientemente obtenido TgShSp1. El aislado TgShSp1 proliferó menos y presento un retraso en la formación de placas de lisis en comparación con el aislado TgME49, pero forma estructuras similares a quistes *in vitro*. No se observó mortalidad en ratones adultos tras la infección con 1-10^5 taquizoitos por vía intraperitoneal o con 25-2000 ooquistes por vía oral del aislado TgShSp1. En ovejas infectadas por vía oral con ooquistes, la infección con el aislado TgME49 produjo un incremento ligeramente mayor en las temperaturas rectales y una mayor carga parasitaria en los cotiledones de ovejas que paren y en el cerebro sus respectivos corderos, pero no se observaron diferencias entre estos dos aislados en la mortalidad perinatal ni en las lesiones o número de corderos positivos a *T. gondii*. La infección congénita tras el desafío a mitad de la gestación con el aislado TgShSp1, evaluada mediante la mortalidad perinatal y la transmisión vertical, fue diferente en función de la especie desafiada. En ratones, se observó una mortalidad del 50% de las crias cuando la madre se desafió con una dosis alta de ooquistes (500 ooquistes del aislado TgShSp1), mientras que en ovejas infectadas con la misma dosis de ooquistes se observó la mortalidad de todos los fetos. Asimismo una mortalidad del 9% y 27% de las crías se encontró en ratones tras la infección con 100 y 25 ooquistes del aislado TgShSp1, respectivamente, mientras que en ovejas, la infección con 50 y 10 ooquistes del aislado TgShSp1 desencadenó mortalidad en el 68% y 66% de los fetos/corderos. Tan solo se encontraron diferencias en transmisión vertical tras la infección con dosis bajas de ooquistes (100% tras la infección con 10 ooquistes del aislado TgShSp1 en ovejas y 37% en ratón tras la infección con 25 ooquistes del aislado TgShSp1). En conclusión, la virulencia en ratones con aislados tipo II de *T. gondii* quizás no sea un buen indicador para predecir el resultado de la infección en ovejas gestantes.

Virulence in mice of a *Toxoplasma gondii* type II isolate does not correlate with the outcome of experimental infection in pregnant sheep

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Submitted to "Frontiers in Cellular and Infection Microbiology", research topic "*Toxoplasma gondii* Host Interactions: A Story of Immune Attack and Parasite Counterattack" on day 18th September 2018.

Photograph courtesy of Luis Miguel Ferrer

Abstract

Toxoplasma gondii is an apicomplexan parasite that infects almost all warm-blooded animals. Little is known about how the parasite virulence in mice extrapolates to other relevant hosts. In the current study, *in vitro* phenotype and *in vivo* behavior in mice and sheep of a type II *T. gondii* isolate (TgShSp1) were compared with the reference type II *T. gondii* isolate (TgME49). The results of *in vitro* assays and the intraperitoneal inoculation of tachyzoites in mice indicated an enhanced virulence for the laboratory isolate, TgME49, compared to the recently obtained TgShSp1 isolate. TgShSp1 proliferated at a slower rate and had delayed lysis plaque formation compared to TgME49, but it formed more cyst-like structures *in vitro*. No mortality was observed in adult mice after infection with $1\text{-}10^5$ tachyzoites intraperitoneally or with 25-2000 oocysts orally of TgShSp1. In sheep orally challenged with oocysts, TgME49 infection resulted in sporadically higher rectal temperatures and higher parasite load in cotyledons from ewes that gave birth and brain tissues of the respective lambs, but no differences between these two isolates were found on perinatal mortality or lesions and number of *T. gondii*-positive lambs. The congenital infection after challenge at mid-pregnancy with TgShSp1, measured as perinatal mortality and vertical transmission, was different depending on the challenged host. In mice, mortality in 50% of the pups was observed when a dam was challenged with a high oocyst dose (500 TgShSp1 oocysts), whereas in sheep infected with the same dose of oocysts, mortality occurred in all fetuses. Likewise, mortality of 9% and 27% of the pups was observed in mice after infection with 100 and 25 TgShSp1 oocysts, respectively, while in sheep, infection with 50 and 10 TgShSp1 oocysts triggered mortality in 68 and 66% of the fetuses/lambs. Differences in vertical transmission in the surviving offspring were only found with the lower oocyst doses (100% after infection with 10 TgShSp1 oocysts in sheep and only 37% in mice after infection with 25 TgShSp1 oocysts). In conclusion, virulence in mice of *T. gondii* type II isolates may not be a good indicator to predict the outcome of infection in pregnant sheep.

Keywords: *Toxoplasma gondii*; type II; virulence; phenotypic traits; mice; sheep; congenital toxoplasmosis

1. Introduction

Toxoplasma gondii is an apicomplexan parasite capable of infecting almost all warm-blooded animals and causing potentially fatal disease in humans and some relevant domestic species, such as small ruminants (Dubey, 2010). *T. gondii* may be transmitted horizontally by oral ingestion of infectious oocysts from the environment and tissue cysts contained in raw and undercooked meat or vertically by transplacental transmission of tachyzoites (Tenter *et al.*, 2000). *T. gondii* diverges in three main clonal lineages, I, II and III, with marked differences in mouse virulence (Howe and Sibley, 1995). Type I isolates are highly virulent in mice, whereas types II and III show a dose-dependent mortality (Saeij *et al.*, 2006). Laboratory isolates from types I, II and III are used in *T. gondii* research and have been maintained in successive passages in cell culture and mice. Enhanced virulence throughout successive passages in cell culture and mice for *T. gondii* type I isolates (e.g., RH isolate) has been widely reported (Villard *et al.*, 1997; Mavin *et al.*, 2004; Khan *et al.*, 2009b), but little is known about the influence of continuous passages in type II isolates. Likewise, there is little information on how *T. gondii* virulence in mice compares to virulence

in other species, particularly in those experiencing clinical toxoplasmosis, such as sheep and humans.

Transmission of *T. gondii* from dams to offspring during pregnancy (congenital toxoplasmosis) is one of the consequences of infection (Innes *et al.*, 2009; McAuley, 2014). Many animal experimental models have been developed; among them, mouse models are the most frequently used due to similarities in placental histology between rodents and humans (Vargas-Villavicencio *et al.*, 2016). However, the structure of the placenta, reproductive physiology and immune responses greatly differ between rodents and ruminants, which clearly could influence the passing of *T. gondii* through the maternofetal interface and the modulation of the host immune responses during pregnancy (Entrican, 2002). In sheep, *T. gondii* is one of the main abortifacient agents (Dubey, 2009b). Type II *T. gondii* isolates are the most prevalent in all of the hosts in Europe, including sheep (Chessa *et al.*, 2014). In most of the experimental studies in pregnant sheep, type II isolates M1, M3 and M4 have been used (Dubey, 2009b; Castaño *et al.*, 2014), but the virulence in mice of these isolates was never assessed.

The aim of this study was to compare the phenotype *in vitro* and the virulence in mice of a newly obtained *T. gondii* type II isolate (TgShSp1) with that of the laboratory type II reference isolate (TgME49) and to compare congenital infection in mice and in sheep.

2. Materials and methods

2.1. Ethics statement

Animal procedures for the *T. gondii* isolation by a mouse bioassay of field samples from sheep abortions (PROEX 274/16), for evaluation of virulence by intraperitoneal inoculation of tachyzoites in non-pregnant mice (PROEX 274/16) and for *T. gondii* infection of mice and cats (PROEX 166/14) for oocyst production were approved by the Animal Welfare Committee of the Community of Madrid, Spain, following proceedings described in Spanish and EU legislation (Law 32/2007, R.D. 53/2013, and Council Directive 2010/63/EU). Animal procedures to characterize TgShSp1 oocysts in pregnant mice were approved by the Animal Welfare Committee of the Canton of Bern (approval No. BE 101/17). All sheep handling practices were approved by the local government and followed the recommendations of the Directive 2010/63/EU of the European Parliament, the Council on the protection of animals used for scientific purposes, and the IGM-CSIC Animal Experimentation Committee (protocol number 416-2016). All animals used in this study were handled in strict accordance with good clinical practices, and all efforts were made to minimize suffering.

2.2. *T. gondii* isolates, isolation of the TgShSp1 and genotyping

The *T. gondii* type II ovine isolate TgME49 (genotype #1) isolated in 1958 from sheep muscle (Lunde and Jacobs, 1983) was kindly donated by Dr. J.C. Boothroyd and had an unknown passage number, but it had been routinely maintained in cell culture and mice. The *T. gondii* type II (genotype #3) isolate (TgShSp1) was obtained from a *T. gondii* ovine abortion outbreak in a Spanish sheep flock (Assaf breed) in the province of Palencia (northwest Spain), which suffered abortion in 30 out of 239 pregnant sheep (12.5%).

TgShSp1 was isolated by passage in mice from a brain of an ovine aborted fetus, in which *T. gondii* infection was confirmed by PCR within 24 h after collection as described (Regidor-Cerrillo *et al.*, 2008). Briefly, fetal brain (6 g) was homogenized in 6 mL of PBS containing 2% antibiotic-antimycotic solution (Gibco, Thermo Fisher Scientific, Waltham, MA, USA), filtered in sterile gauze, and centrifuged at 1350 g for 15 min. The supernatant was discarded, and the sediment was suspended in 1400 µL of PBS with antibiotic-antimycotic solution, and 400 µL was inoculated subcutaneously into one 8-

week-old female CD1 mouse that was followed for clinical signs. At day 40 pi, asymptomatic mouse were euthanized, and the brain collected and tested by PCR (see below). A fraction of the PCR-positive mouse brain was homogenized in PBS with antibiotics by passing through a descending series of needles (20 to 25 G) and immediately subcutaneously inoculated into two other CD1 mice. At 11 dpi, mice were euthanized, and peritoneal flushes were used for isolation in MARC-145 cell culture (Regidor-Cerrillo *et al.*, 2008) and checked for the presence of parasite by PCR.

The *T. gondii* isolate obtained was genotyped by polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) using 12 molecular markers (SAG1, 3'-SAG2, 5'-SAG2, Alt.SAG2, SAG3, BTUB, GRA6, c22-8, c29-2, L358, PK1 and Apico) as previously described Su *et al.* (2006). The digested PCR products were visualized by 2.5% agarose gel electrophoresis, stained with Gel Red® Nucleic Acid Gel Stain (Biotium®, Fremont, California, USA), observed under UV light, assigned to a *T. gondii* type and classified according to genotypes present in ToxoDB (http://toxodb.org/toxo/).

2.3. *In vitro* assays

MARC-145, Vero and Human Foreskin Fibroblasts (HFF) cell lines were used for studying the tachyzoite-to-bradyzoite conversion and proliferation of *T. gondii* isolates in vitro. Cells were routinely maintained in DMEM (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) with phenol red supplemented with 10% heat-inactivated, sterile, filtered fetal calf serum (FCS) (Gibco, Thermo Fisher Scientific, Waltham, MA, USA), 2 mM glutamine (Lonza Group, Basel, Switzerland) and a mixture of penicillin (100 U/ml), streptomycin (100 µg/ml) and amphotericin B (Lonza Group, Basel, Switzerland) at 37 °C in a humidified atmosphere of 5% CO_2. TgME49 and TgShSp1 were maintained by serial passages in MARC-145 and Vero cells in the same culture medium with 2% FCS. Tachyzoites used for in vitro assays were harvested 3 days pi (TgME49) or 5 days pi (TgShSp1, passage 10), when the majority of parasites were still intracellular and purified by a PD-10 column (GE Healthcare, Little Chalfont, United Kingdom), as described (Regidor-Cerrillo *et al.*, 2011). Tachyzoite viability was confirmed by trypan blue exclusion, and numbers were determined by counting in a Neubauer chamber. All assays were carried out in triplicate, including at least three replicates in each assay.

2.3.1. Evaluation of the tachyzoite-to-bradyzoite differentiation in vitro through cyst wall-specific DBL staining

Purified *T. gondii* parasites of the TgME49 or TgShSp1 isolate (2 x 10^3 tachyzoites) were added to MARC-145 monolayers grown to confluence in 24-well plates. At 24 h, culture medium was replaced by DMEM with alkaline pH (8-8.2), and plates were incubated at 37°C without CO_2 supplementation for 3-4 days (Skariah *et al.*, 2010). Duplicate plates were maintained for 3 days under regular conditions (pH 7.2-7.4 and 5% CO_2). Tachyzoite-to-bradyzoite conversion was evaluated at 72-96 h by double immunofluorescence staining, in fixed cell monolayers with paraformaldehyde 3% and glutaraldehyde 0.05% using a polyclonal mouse-anti *T. gondii* antiserum at a dilution of 1:100 as primary antibody, Alexa Fluor® 594 Goat Anti-Mouse IgG (H + L) (Life technologies, Carlsbad, CA, USA) at a dilution of 1:1000 as secondary antibody for parasite staining, and the *Dolichos biflorus* lectin (DBL) (Vector Labs, Burlingame, United States) at a dilution of 1:50 for cyst wall staining. Cell nuclei were stained with DAPI. Finally, the total numbers of DBL-positive cysts and DBL-negative structures compatible with parasite structures, including lysis plaques, were counted using an inverted fluorescence microscope (Nikon Eclipse TE200) at 200x magnification. The percentage of conversion for each well was determined.

2.3.2. *In vitro* intracellular proliferation assays

T. gondii proliferation was evaluated in Vero cells by a plaque assay and in HFF determining the tachyzoite yield at 48 h pi. For this assay, Vero cultures grown to confluence in 24-well plates were infected with 5 x 10^4 purified tachyzoites of either TgME49 or TgShSp1 and further maintained at 37 °C and 5% CO_2 for 4 days and stained with 0.2% crystal violet (Alfa Aesar, Haverhill, Massachusetts, United States) solution in 2% ethanol. (Ufermann *et al.*, 2017). Images were captured using a SMZ1000 binocular loupe (Nikon®, Tokyo, Japan).

Tachyzoite yield in HFF was determined by quantifying the number of tachyzoites at 48 h pi (TY_{48h}) by real-time PCR (qPCR). HFF cultures grown to confluence in 24-well plates were infected with 10^5 purified *T. gondii* tachyzoites and maintained for 48 h at 37°C in 5% CO_2 as previously described Regidor-Cerrillo *et al.* (2011). Then, the medium was removed, and cells were recovered in 150 µL lysis buffer and 10 µL proteinase K (Macherey-Nagel, Düren, Germany) for DNA extraction and quantification of parasite genomic DNA by qPCR.

2.4. *In vivo* experimental infections

2.4.1. Virulence assessment in non-pregnant mice intraperitoneally inoculated with TgShSp1 and TgME49 tachyzoites

TgShSp1 and TgME49 virulence was determined in mice following recommendations for standardization described by Saraf *et al.* (2017). For the *in vivo* challenge, tachyzoites of TgShSp1 (passage 10) and TgME49 (unknown passage number) were recovered from Vero cultures when they were still largely intracellular (> 80% of undisrupted parasitophorous vacuoles) (Regidor-Cerrillo *et al.*, 2010), repeatedly passed through a 27-gauge needle at 4°C and filtered through a 5-µm polycarbonate filter (IpPORE®, IT4IP, Louvain-la-Neuve, Belgium) (Saraf *et al.*, 2017). The tachyzoite viability was determined by Trypan blue exclusion and counted in a Neubauer chamber. Tachyzoite 10-fold serial dilutions were performed starting from 10^5 to 1 tachyzoite(s) suspended in 200 µL of PBS, and each dilution was inoculated intraperitoneally into five 8-week-old female CD1 mice (Janvier-Labs, Laval, France) within 30 min of harvesting the parasites from cell culture. Five control female mice were inoculated with PBS. Mice were observed daily, and clinical signs (morbidity) were scored according to the description made by Arranz-Solis *et al.* (2015a). Briefly, scores were classified as 0 (no alterations), 1 (ruffled coat), 2 (rounded back), 3 (noticeable loss of body condition/severe weight loss) or 4 (nervous signs such as activity decrease, hind limb paralysis, walking in circles or head tilt). As a humane endpoint, mice exhibiting evident loss of body condition (score of 3) or nervous signs (score of 4) were culled to limit unnecessary suffering. Mice with clinical scores of 0, 1 and 2 were euthanized at six weeks p.i. Samples of blood were collected from mice for serology by IFAT and brain and lung for parasite detection by PCR.

2.4.2. Generation, purification and sporulation of *T. gondii* oocysts

Oocysts of the TgShSp1 isolate were obtained through oral infection of cats as previously described Müller *et al.* (2017c). Briefly, ten 8-week-old female CD1 mice (Janvier-Labs, Laval, France) were inoculated intraperitoneally with 10^5 tachyzoites of TgShSp1 (passage 10). At 2 months postinoculation, mice were euthanized, and the brains were collected. Two twelve-week-old kittens free of *T. gondii* and other relevant feline pathogens (Isoquimen S.L., Barcelona, Spain) were fed a pool of 5 brains each. Feces were collected from kittens daily and examined to detect shedding of *T. gondii* oocysts. Unsporulated oocysts were harvested from feces and sporulated by resuspending in 2% H_2SO_4 for 4 days at room temperature.

Sporulated oocysts were kept at 4 °C until used. The same batch of sporulated oocysts of the TgME49 and TgShSp1 was used in mouse and sheep infections. Sporulated oocysts of TgME49 originated from the same batch as described earlier (Müller *et al.*, 2017c).

2.4.3. Assessment of TgShSp1 oocyst infection in pregnant mice

TgShSp1 was evaluated in pregnant mice out similarly as previously described for TgME49 (Müller *et al.*, 2017c). CD1 females (50 mice) and males (25 mice) were purchased from Charles River Laboratories (Sulzberg, Germany) at the age of 8 weeks and were maintained in a common room under conventional day/night cycle housing conditions. Females at 9 weeks of age were synchronized with respect to estrus and were distributed into cages, where two females and one male were housed together for 3 days (during which 3 females died). Subsequently, the female mice were orally infected by gavage with high doses of oocysts: 2000 oocysts (group A, n = 9) and 500 oocysts (group B, n = 9), an intermediate dose of oocysts: 100 oocysts (group C, n = 10) and a low dose of oocysts: 25 oocysts (group D, n = 10) suspended in 100 µL of carboxymethyl cellulose solution (0.5% in water) at day 7 postmating. The control group (group E, n = 9) received carboxymethyl cellulose solution alone. Pregnancy was confirmed 2 weeks postmating by weighing, and pregnant mice were then allocated into single cages to give birth on days 19–22 and to rear their pups for an additional 4 weeks. During this time, those females that had remained non-pregnant were maintained in cages of three to five mice. Dams and their offspring were evaluated daily from birth to day 28 postpartum (pp). Despite the numerous parameters evaluated, pup mortality (number of pups born dead or euthanized due to severe clinical signs) and vertical transmission (surviving pups being PCR-positive in the brain) were the most relevant assessments. Data on pregnancy rate (percentage of female mice housed with males that became pregnant), litter size (number of delivered pups per dam) and clinical signs (morbidity) of dams and non-pregnant mice were recorded during this time as described above for intraperitoneal inoculation. Neonates were weighed every second day from day 14 pp until the end of the experiment (day 28 pp) to evaluate morbidity in the offspring. Day 14 pp was chosen as a starting point for weight monitoring to avoid excessive handling of the pups during the first 2 weeks after birth, which can result in rejection by the dams. Dams, non-pregnant mice and pups were euthanized in a CO_2 chamber at 28 days pp. Blood from dams and non-pregnant mice was recovered by cardiac puncture, and sera were obtained to test humoral immune responses. Brains and lungs were removed from dams and non-pregnant mice and stored at -20°C until determination of parasite load. The heads of pups that survived were collected and stored at -20°C. Subsequently, the frozen heads were cleaved and brains were removed. The frozen brains from pups were immediately processed for DNA purification and then parasite quantification. Whenever possible, dead pups succumbing to the infection early after birth were removed, their heads sampled and their brains analyzed.

2.4.4. Assessment of TgShSp1 and TgME49 oocyst infections in pregnant sheep

Fifty-four pure Rasa Aragonesa breed female ewes aged 12 months were selected from a commercial flock. All animals were seronegative for *T. gondii*, *N. caninum*, border disease virus (BDV), Schmallenberg virus (SBV), *Coxiella burnetii* and *Chlamydia abortus* as determined by enzyme-linked immunosorbent assay (ELISA). They were estrus-synchronized and mated with pure-bred Rasa Aragonesa tups for 2 days, after which the rams were separated from the ewes. Pregnancy and fetal viability were confirmed by ultrasound scanning (US) on day 40 postmating. Pregnant ewes (n = 37) were randomly distributed into seven experimental groups and housed at the Instituto de Ganadería

de Montaña (CSIC-Universidad de León), León, Spain.

Thirty-three pregnant ewes were orally dosed on day 90 of pregnancy with a high dose of oocysts (500 sporulated oocysts) of TgShSp1 (group 500A, G500A, n=6) or TgME49 (group 500B, G500B, n=5), an intermediate dose of oocysts (50 oocysts) of TgShSp1 (group 50A, G50A, n=6) or TgME49 (group 50B, G50B, n=5) or a low dose of oocysts (10 oocysts) of TgShSp1 (group 10A, G10A, n=6) or TgME49 (group 10B, G10B, n=5). The four remaining sheep were used as negative controls of infection (uninfected) and received 50 mL of PBS on day 90 of pregnancy.

Pregnant ewes were observed daily throughout the experimental period. Rectal temperatures were recorded daily from day 0 until 14 days pi and then weekly to evaluate morbidity. The physiological range for rectal temperatures in sheep was obtained from Diffay *et al*. (2002), and rectal temperatures above 40°C were considered hyperthermic. Fetal viability was assessed by US monitoring of fetal heartbeat and movements twice a week after infection. When fetal death occurred, or immediately after parturition, dams and lambs were first sedated with xylazine (Rompun, Bayer, Mannheim, Germany) and then euthanized by an intravenous overdose of embutramide and mebezonium iodide (T61, Intervet, Salamanca, Spain). Animals from the uninfected group were examined by US every two weeks.

According to the perinatal survival, sheep were classified into three categories: (a) suffering early abortions (i.e., between 8 and 11 dpi); (b) suffering late abortions, which occurred from 12 to 50 dpi; and c) sheep delivering stillbirths, mummified fetuses or live lambs from 51 dpi. Perinatal mortality was calculated in each group considering fetal mortality during pregnancy and stillbirths according to Hubbert et al. (1972). After birth, lambs were clinically inspected and then sedated and euthanized. Lambs showing weakness in relation to all live lambs were used to calculate morbidity in the offspring. In spite of the numerous parameters evaluated, perinatal mortality and vertical transmission in live lambs (seropositivity and parasite detection in brain or lung) were the most relevant assessments.

Blood samples to evaluate humoral immune responses were collected prior to infection, at 3, 5, 7 and 10 days pi and then weekly by jugular blood draw. Precolostral serum was collected from lambs immediately after delivery from dams. To prevent any transmission of colostral antibodies from dams, udders were covered with a piece of cloth one week before the expected date of delivery as a preventive measure, and lambs were separated from their mothers immediately after birth. Serum samples were stored at −80°C until analysis.

During necropsy, six randomly selected placentomes or cotyledons from aborted dams and dams that gave birth, respectively, were recovered from each placenta, transversally cut into 2–3 mm-thick slices, and fixed in 10% formalin for histopathological examination, whereas the remaining tissues from these placentomes/cotyledons were stored at −80°C for further DNA extraction and PCR analyses. Samples from fetal tissues, including brain and lungs, were stored at −80°C for DNA extraction or were fixed in 10% formalin for histopathology. Thoracic and abdominal fluids were also collected from fetuses and stillborn lambs from which precolostral sera could not be obtained, and maintained at −80°C for serology.

2.5. Serological analyses: IFAT and ELISA

The serum samples from mice used for isolation and determination of virulence were analyzed by the immunofluorescence antibody test (IFAT) for the detection of anti-*T. gondii* IgG as previously described Alvarez-Garcia *et al.* (2003), using an anti-mouse IgG conjugated

to FITC (Sigma-Aldrich, Madrid, Spain) diluted 1:64 in Evans Blue (Sigma-Aldrich). We used the cut-off of 1:25. Serum titers for *T. gondii* in oocyst-infected mice were assessed by ELISA as previously described for *Neospora caninum*-infected mice (Debache *et al.*, 2008; Debache *et al.*, 2009), except that soluble antigen extract from *T. gondii* tachyzoites was used (Alaeddine *et al.*, 2005).

T. gondii-specific IgG antibody levels in sheep were measured using an in-house indirect ELISA similarly as previously described Castaño *et al.* (2014). First, 96-well microtiter plates (Thermo Fisher Scientific, Waltham, USA) were coated with 100 µL soluble *T. gondii* antigen (1.5 µg/mL in 100 mM carbonate buffer pH 9.6) overnight at 4°C. Plates were blocked, and serum samples were diluted 1:100 using 3% bovine serum albumin diluted in PBS containing 0.05% Tween 20 (PBS-T). Subsequently, horseradish peroxidase-conjugated protein G (Sigma-Aldrich, Madrid, Spain) diluted 1:3000 in PBS-T was added. After that, ABTS (Roche, Basilea, Switzerland) was used as substrate. The reaction was stopped adding 100 µL of 0.3 M oxalic acid, and the optical density (OD) was read at 405 nm (OD_{405}). For each plate, values of the OD were converted into a relative index percentage (RIPC) using the following formula: RIPC = (OD_{405} sample – OD_{405} negative control)/(OD_{405} positive control–OD_{405} negative control) × 100. A RIPC value ≥10 indicated a positive result.

The indirect fluorescent antibody test (IFAT) was used to detect specific IgG anti-*Toxoplasma* antibodies in fetal fluids and precolostral sera, adapting the technique previously described for IFAT analysis in *N. caninum*-infected animals (Alvarez-Garcia *et al.*, 2003), using an anti-sheep IgG (Sigma-Aldrich) diluted 1:200 in Evans blue (Sigma-Aldrich). Fetal fluids and precolostral sera were diluted at two-fold serial dilutions in PBS starting at 1:8 (for fetal fluids) and 1:50 (for precolostral sera) up to the endpoint titer. Continuous tachyzoite membrane fluorescence at a titer ≥ 8 for fetal fluids or ≥ 50 for precolostral sera was considered a positive reaction.

2.6. DNA extraction and PCR for parasite detection and quantification in tissues

Genomic DNA from *in vitro* samples was extracted from these samples using the NucleoSpin® DNA RapidLyse Kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. DNA concentrations were adjusted to 20 ng/µL and quantified using qPCR with primer pairs for the 529-bp repeat element for *T. gondii* for parasite quantification and primer pairs for the 28S rRNA gene for quantify cell DNA under conditions previously described (Castaño *et al.*, 2016) and (Collantes-Fernández *et al.*, 2002), respectively.

Genomic DNA was extracted from mice that were used for isolation and determination of virulence out using the commercial Maxwell® 16 Mouse Tail DNA Purification Kit. The *T. gondii* DNA detection was carried out by an ITS-1 PCR adapted to a single tube as previously described Castaño *et al.* (2014). DNA extraction and qPCR analysis from oocyst-infected mice were performed as previously described Müller *et al.* (2017c).

In sheep, genomic DNA was extracted from three 50–100 mg samples taken from each location: six placentomes in aborted dams or six cotyledons in dams that gave birth, as well as fetal brain and lung, using the commercial Maxwell® 16 Mouse Tail DNA Purification Kit. *T. gondii* DNA detection was carried out by an ITS-1 PCR as described above (Castaño *et al.*, 2014). DNA that tested positive by nested-PCR was adjusted to 20 ng/µL and quantified using qPCR as previously described Castaño *et al.* (2014). Parasite number in tissue samples (parasite burden) was expressed as parasite number/mg ovine tissue. Standard curves for *T. gondii* and sheep DNA showed an

average slope of −3.44 and −3.30, respectively, and an $R^2 > 0.99$. Parasite-negative DNA samples were included in each round of DNA extraction and PCR as negative controls.

2.7. Histological processing

After fixation for five days, placental and fetal sheep tissues were cut coronally, embedded in paraffin wax and processed by standard procedures for hematoxylin and eosin (HE) staining. Conventional histological evaluation was carried out on all sections. To quantify the lesions in the brain of stillborn lambs and live lambs, the number and size of glial foci, as well as the total area of lesion in the examined tissue, were calculated through a computer-assisted morphometric analysis on HE-stained sections following the procedure described previously (Arranz-Solis *et al.*, 2015b).

2.8. Statistical analysis

The growth rate and percentage of DBL-positive cysts *in vitro* of TgME49 and TgShSp1 were compared using the Mann–Whitney test. In pregnant mice, differences in seroconversion, pregnancy rates, litter size, pup mortality, and parasite presence in tissues were analyzed by the χ^2 test or Fisher's exact F-test. One-way ANOVA followed by Tukey's multiple comparisons test were employed to compare body weights. Parasite burdens and anti-*T. gondii* antibody levels were analyzed using the nonparametric Kruskal–Wallis test followed by Dunn's test for comparisons between groups, as well as the Mann–Whitney test for pairwise comparisons.

In pregnant sheep, the number of fetuses/lambs suffering perinatal mortality and the number of weak lambs (morbidity) were compared using the χ^2 test or Fisher's exact F-test. Rectal temperatures and humoral immune responses were analyzed using one-way ANOVA followed by Tukey's multiple comparisons test until 14 days pi or until the end of the experiment. Differences in frequency of PCR detection of parasite DNA and in the percentage of cases showing lesions were evaluated using the χ^2 test or Fisher's exact F-test. Differences in parasite burdens and histological measurements of lesions were analyzed using the nonparametric Kruskal–Wallis test followed by Dunn's test for comparisons between groups, as well as the Mann–Whitney test for pairwise comparisons.

Differences between mice and sheep in the number of fetuses/pups/lambs that died in relation to the total number of fetuses/pups/lambs or in the number of surviving offspring infected with *T. gondii* in relation to all live offspring were assessed using the χ^2 test or Fisher's exact F-test. Likewise, a categorization of the parameters to evaluate congenital infection was done, into high (>67%), medium (66-34%), low (<33%) or none (0%) of the fetuses/pups/lambs with clinical signs, perinatal mortality or vertical transmission. Statistical significance for all analyses was established at $P < 0.05$. All statistical analyses were performed using GraphPad Prism 6.01 software (San Diego, CA, USA).

3. Results

3.1. Isolation of the *T. gondii* isolate TgShSp1 from a sheep flock in Spain

On day 40 post-inoculation, one mouse inoculated with the brain homogenate of the sheep abortion was *T. gondii* PCR-positive in the brain. The peritoneal fluid of one of the other two mice inoculated with the positive mouse brain was PCR-positive on day 11 pi. Four days after inoculating the PCR-positive peritoneal flush into cell culture, the isolation of TgShSp1 was confirmed. Genotyping classified TgShSp1 as type II, genotype #3 (ToxoDB).

3.2. TgShSp1 and TgME49 differ in behavior *in vitro*

3.2.1. TgShSp1 exhibits a high capacity to form cysts *in vitro*

At difference in TgME49 free-floating cyst-like structures was often identified by light microscopy in the TgShSp1 infected cultures at 3 days p.i. in successive passages. TgShSp1 cultured under regular conditions (at a neutral pH) demonstrated spontaneous conversion to bradyzoite with a statistically higher number of DBL-positive cysts (14%) compared to TgME49 (2%) ($P < 0.0001$). Additionally, after induction of bradyzoite development (at a basic pH), TgShSp1 formed a higher number of DBL-positive cysts (55%) compared to TgME49 (33%) ($P < 0.0001$) (Figure 1A).

3.2.2. TgME49 shows a higher growth rate *in vitro* than TgShSp1

Evaluation of parasite growth in Vero cells by a lysis plaque assay showed that TgME49 produced large clear zones due to host cell lysis, while during the same period, TgShSp1 showed essentially an intact monolayer cell (Figure 1B). Determination of the TY_{48h} in HFF was also assessed to confirm differences in parasite growth. The TY_{48h} values for TgME49 were significantly higher compared to those from TgShSp1 ($P < 0.0001$) (Figure 1C).

3.3. TgShSp1 and TgME49 differ greatly in virulence in mice

A summary of clinical signs, serology and parasite detection in mice is shown in Table S1. Most of the mice inoculated with doses from 10^5 to 10^2 tachyzoites of TgShSp1 only exhibited a ruffled coat between days 4 and 13 pi, but they did not have to be euthanized due to severe clinical signs (Figure 2A) (Table S1). In contrast, upon infection with TgME49, several mice had to be euthanized due to clinical scores (Figure 2B). The surviving mice infected with doses from 10^5 to 10 tachyzoites of TgME49 exhibited clinical signs (rounded back) between days 8 and 14 pi (Table S1). The LD_{50} for TgME49 was approximately 10^3 tachyzoites *vs.* > 10^5 tachyzoites for TgShSp1.

All mice inoculated with doses from 10^5 to 10 tachyzoites of TgShSp1 were seropositive at 6 weeks pi, with IFAT titers ranging from 1:400 to 1:3200. Concerning mice infected with TgME49, mice euthanized prior to day 24 pi were seronegative, while one mouse infected with 10^3 tachyzoites euthanized on day 33 pi had an IFAT titer of 1:25. Survivors infected with doses from 10^5 to 10 tachyzoites of TgME49 were seropositive at 6 weeks pi, with IFAT titers ranging from 1:50 to 1:800 (Table S1).

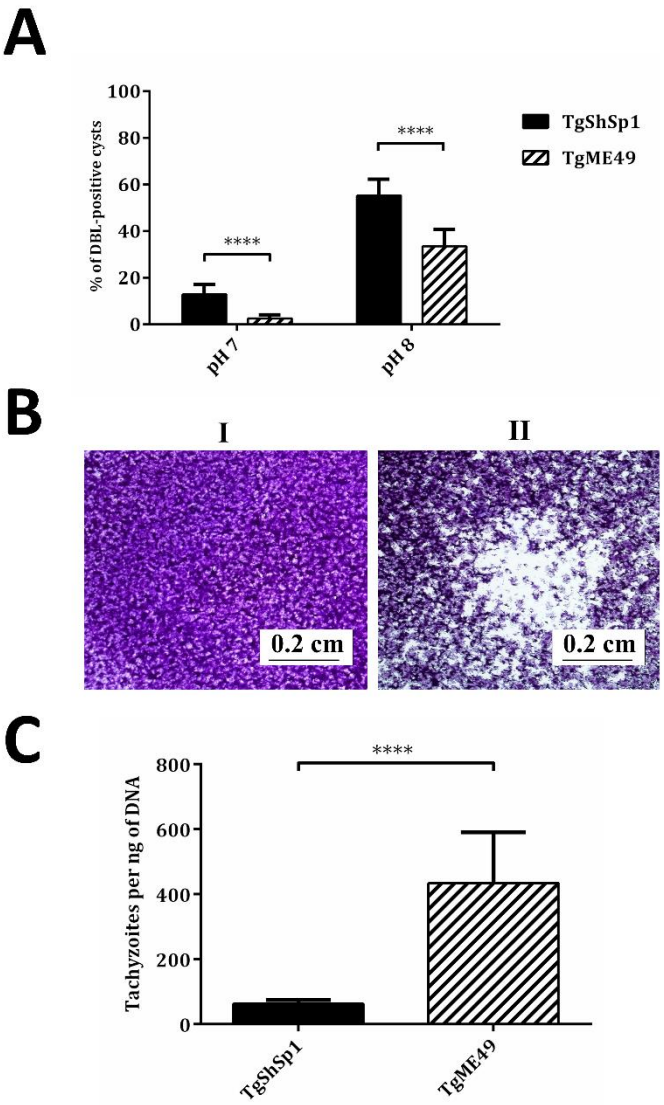
All mice infected with doses from 10^5 to 10 tachyzoites of TgME49 and TgShSp1 were PCR-positive in the brain, confirming *T. gondii* infection. Likewise, almost all lung samples from mice infected with doses from 10^5 to 10 tachyzoites of both isolates were PCR-positive, except lung samples from surviving mice infected with 10 tachyzoites of TgME49. Mice infected with 1 tachyzoite did not show clinical signs, they were seronegative, and all tissue samples were PCR-negative, identical to uninfected mice (Table S1).

3.4. TgShSp1 shows low virulence in mice infected with oocysts but is efficiently transmitted to offspring

3.4.1. Evaluation of TgShSp1 infection in dams

Clinical signs in infected dams were generally mild. Therefore, none of the dams had to be euthanized due to severe clinical signs. At 12 days pi, one out of five dams infected with 2000 oocysts (group A) exhibited ruffled coat (1), and one out of six dams infected with 500 oocysts (group B) displayed rounded back (2). No clinical signs were

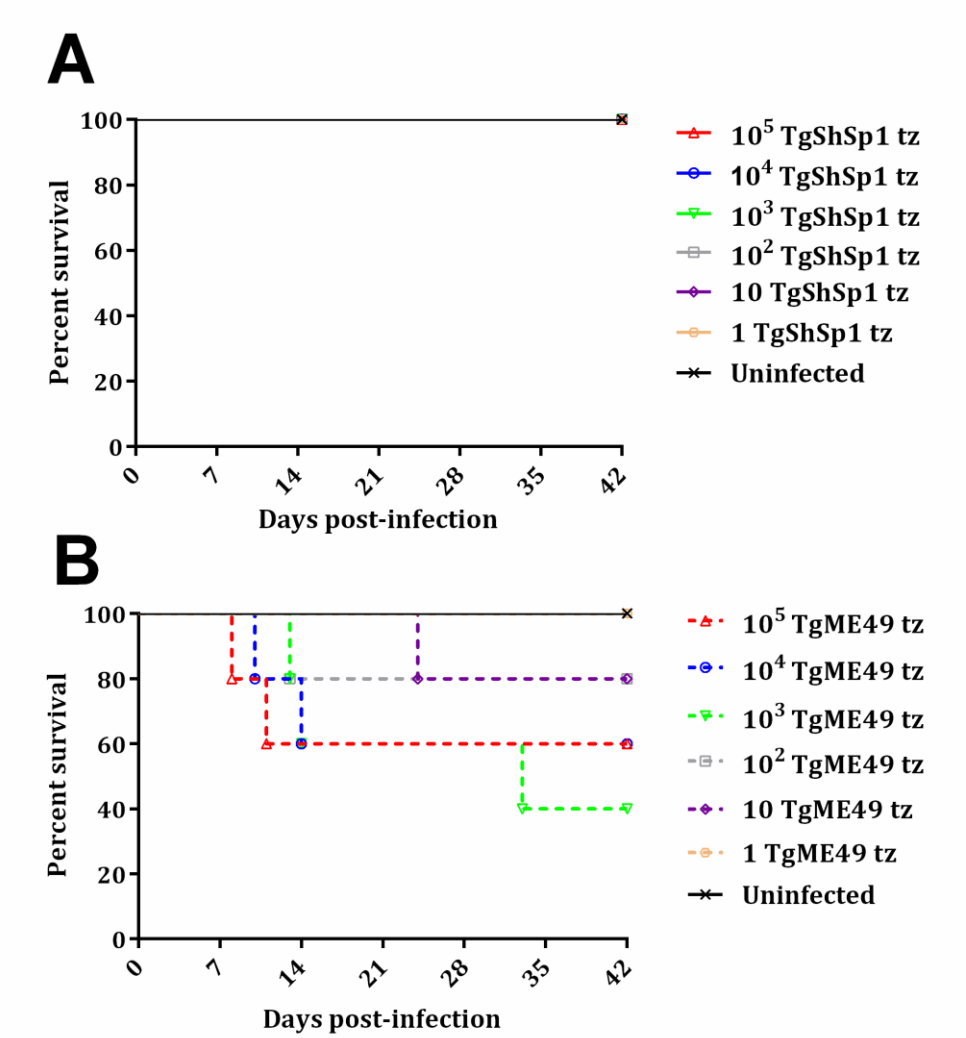
Figure 1-Tachyzoite-to-bradyzoite differentiation, plaque formation and tachyzoite yield *in vitro* of TgME49 and TgShSp1 isolates. (A) *In vitro* tachyzoite-to-bradyzoite differentiation at neutral and alkaline pH. Percentage of events being DBL-positive cysts at pH 7 and pH 8 are represented. The rest of nonrepresented events were found to be DBL-negative structures and lysis plaques. (****) marks the higher spontaneous and induced differentiation into bradyzoites with the TgShSp1 isolate. (B) Plaque formation with TgShSp1 (I) and TgME49 (II) isolates. Plaques are visible as clear zones on a crystal violet-stained Vero monolayer background. (C) A column-plot graph representing the tachyzoite yield (TY_{48h}) of TgME49 and TgShSp1. Values of replicates from experiments performed in triplicate for each isolate. Error bars indicate the SD. (****) marks the significantly higher $TY_{48\,h}$ values for TgME49 compared to TgShSp1.



observed in dams infected with 100, 25 or 0 oocysts (groups C, D and E). Pregnancy rates ranged from 55 to 66%, with no significant differences between them. Similarly, no differences between the groups were found in litter size (11.8–14.4 delivered pups), suggesting that pregnancy was not noticeably altered by infection with TgShSp1 oocysts (Table 1).

All infected dams with 2000, 500 and 100 oocysts (groups A, B and C) developed

Figure 2 - Survival curve of CD1 mice after infection with *T. gondii* tachyzoites of TgShSp1 isolate (A) and TgME49 isolate (B). Five mice per group were infected i.p. with 10^5, 10^4, 10^3, 10^2, 10 or 1 tachyzoite of the TgShSp1 isolate. Survival was monitored for 42 days. Each point represents the percentage of surviving animals at that day, and downward steps correspond to euthanasia due to severe clinical signs.



Toxoplasma-specific humoral immune responses at day 28 pp. However, although only seroconversion in half of the dams was observed in the group infected with 25 oocysts (group D), there were no statistically significant differences in the number of dams showing seroconversion between infected groups (Table 1). Anti-*T. gondii* IgG levels were significantly increased in groups infected with 2000 ($P < 0.05$), 500 ($P < 0.01$) and 100 oocysts ($P < 0.01$) in comparison to the unchallenged group in which all dams were seronegative (Figure S1A). *T. gondii* DNA was detected in the brain of all dams from infected groups, with the exception of three dams in the group infected with 25 oocysts (group D) (Table 1). Quantitative evaluation of parasite burdens in brain showed no significant differences between infected groups (Figure S2A). In the lungs, parasite DNA was detected in the 50-83% of the dams from oocyst-infected groups, without significant differences in parasite detection or parasite load between them (Table 1) (Figure S3A).

Table 1 - Effects of oral infection with TgShSp1 oocysts on infection status in adult mice, fertility in dams, mortality in pups and vertical transmission in surviving offspring.

			Non-pregnant			Pregnant mice			Pups				
Oocysts	**N°**	**Pregnant[a]**	**Lung positive**	**Brain positive**	**Serum positive**	**Lung positive**	**Brain positive**	**Serum positive**	**Litter size (0 days *post partum*)[b]**	**Mortality per litter (28 days *post partum*)[c]**	**Litter with 100% mortality (28 days *post partum*)[d]**	**Surviving (28 days *post partum*)[e]**	**Brain positive (28 days *post partum*)**
2000	9	5/9	0/4	4/4	4/4	3/5	5/5	5/5	62	5/5	1/5	31 (50%)*	31 (100%)
500	9	6/9	0/3	3/3	3/3	5/6	6/6	6/6	71	5/6	2/6	36 (50%)*	36 (100%)
100	10	6/10	3/4	4/4	4/4	5/6	6/6	6/6	83	1/6	0/6	76 (91%)	76 (100%)
25	10	6/10	0/4	3/4	3/4	3/6	3/6	3/6	72	5/6	1/6	53 (73%)	20 (37%)****
Control	9	5/9							72	1/5	0/5	71 (98%)	

[a] Number of pregnant mice/mice housed with males.
[b] Number of full-term delivered pups.
[c] Number of litters with at least one pup born dead or euthanized due to severe clinical signs/total number of litters.
[d] Number of litters in which all delivered pups born dead or have to be euthanized due to severe clinical signs/total number of litters.
[e] Number of pups surviving at day 28 pp (percentage).
(*) indicates $P < 0.05$ and (****) indicates $P < 0.0001$ significant differences.

3.4.2. Evaluation of TgShSp1 infection in offspring mice

Most pups were born dead (82/93; 88%), although a few pups had to be euthanized due to severe clinical signs between day 2 and day 21 pp (11/93; 12%). Half of the pups in the group infected with 2000 oocysts (group A) and with 500 oocysts (group B) were born dead or had to be euthanized due to severe clinical signs and had significantly higher pup mortality compared to the uninfected group ($P < 0.05$) (Table 1). In groups infected with 100 oocysts (group C) and 25 oocysts (group D), respectively, 9% and 27% of pups were born dead or had to be euthanized due to severe clinical signs. Only one of seventy-two pups was born dead in the uninfected group (group E) (Table 1). Starting from day 14 pp, offspring of the uninfected group (group E) showed significantly higher body weight than groups infected with 500 ($P < 0.0001$) and 100 oocysts ($P < 0.001$), and the same was true of those infected with 2000 oocysts (group A) from day 22 pp ($P < 0.001$). However, no decreased body weight was noted in pups infected with 25 oocysts (group D) (Figure 3A).

Vertical transmission was detected upon PCR analyses in all brains (100%) of surviving pups from groups infected with 2000, 500 and 100 oocysts (groups A, B and C). However, parasite was only detected in 37% of the brains from pups infected with 25 oocysts (group D), with significantly lower parasite detection compared to groups infected with 2000, 500 and 100 oocysts ($P < 0.0001$) (Table 1). Pups infected with 25 oocysts (group D) and with 100 oocysts showed lower parasite burden compared to those infected with 2000 and 500 oocysts (groups A and B) ($P < 0.0001$) (Figure 3B). No *T. gondii* DNA could be detected in the brain of pups that had died on day 0 or 1 pp.

3.4.3. Evaluation of TgShSp1 oocyst infection in non-pregnant mice

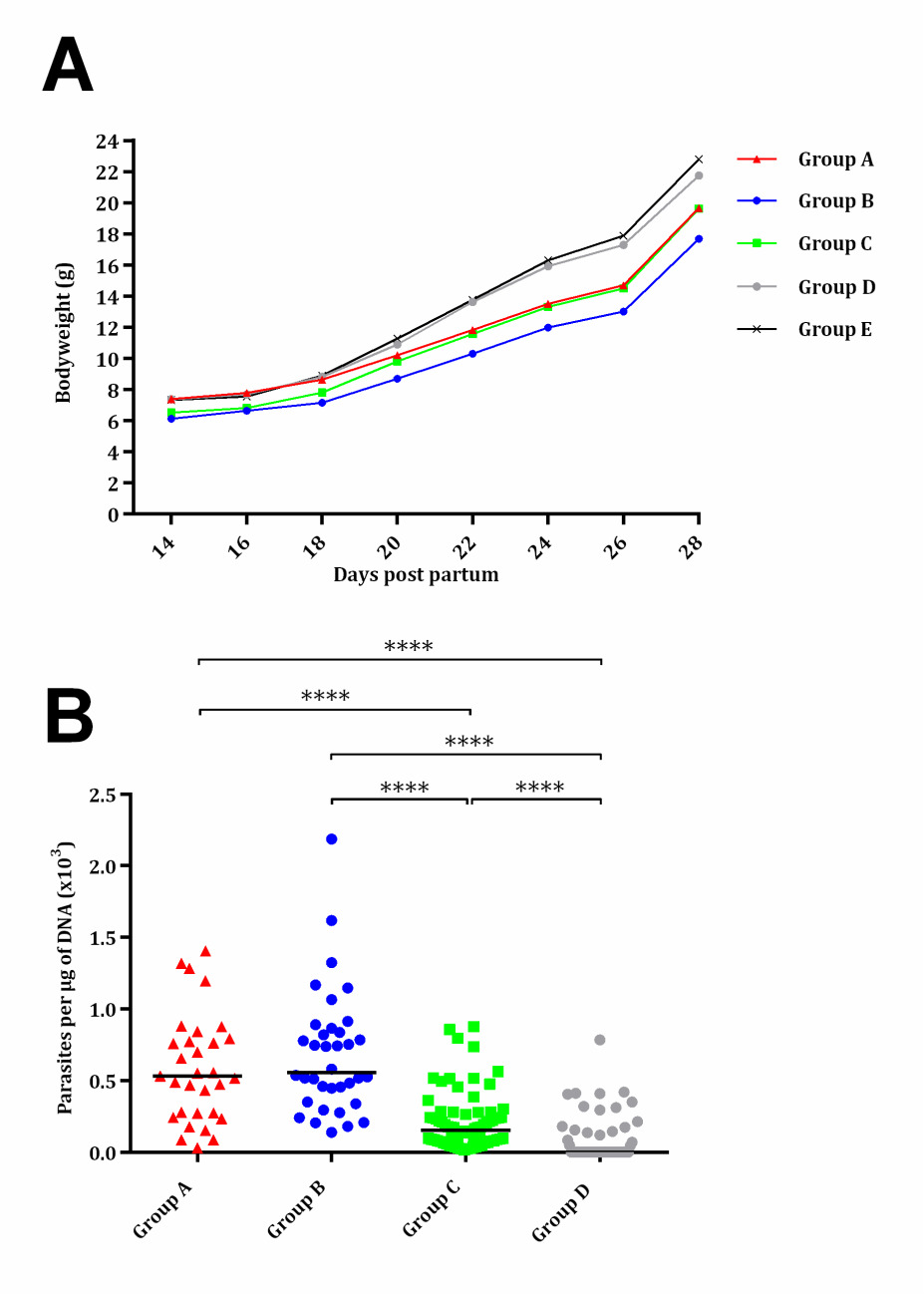
Similarly, to pregnant mice, clinical signs in non-pregnant mice infected with oocysts were generally mild. Therefore, none of the non-pregnant mice had to be euthanized due to severe clinical signs. Only ruffled coat was observed in all mice infected with 2000 oocysts (group A), in one out of three mice in the group infected with 500 oocysts (group B) and in one out of four non-pregnant mice in the group infected with 100 oocysts (group C). All infected non-pregnant mice infected with 2000, 500 and 100 oocysts (groups A, B and C) developed *Toxoplasma*-specific humoral immune responses at day 28 p.p. that were significantly increased in comparison to the unchallenged group, with basal IgG levels ($P < 0.05$) (Figure S1B). In the group infected with 25 oocysts (group D), only three of the four non-pregnant mice seroconverted (Table 1). Further analyses of antibody responses of pregnant and non-pregnant mice in each group did not reveal any significant differences. *T. gondii* DNA was detected in the brains of all non-pregnant mice from infected groups, with the exception of one mouse infected with 25 oocysts (group D) (Table 1). In the lungs, parasite DNA was only detected in three out of four samples from mice infected with 100 oocysts (group C). Quantitative evaluation showed no significant differences in parasite load in non-pregnant mice (Figures S2B and S3B). The comparison of parasite load in brain and lungs in pregnant mice *vs* non-pregnant mice revealed no significant differences.

3.5. TgShSp1 and TgME49 oocyst infection cause similar perinatal mortality and vertical transmission in pregnant sheep

3.5.1. Clinical observations

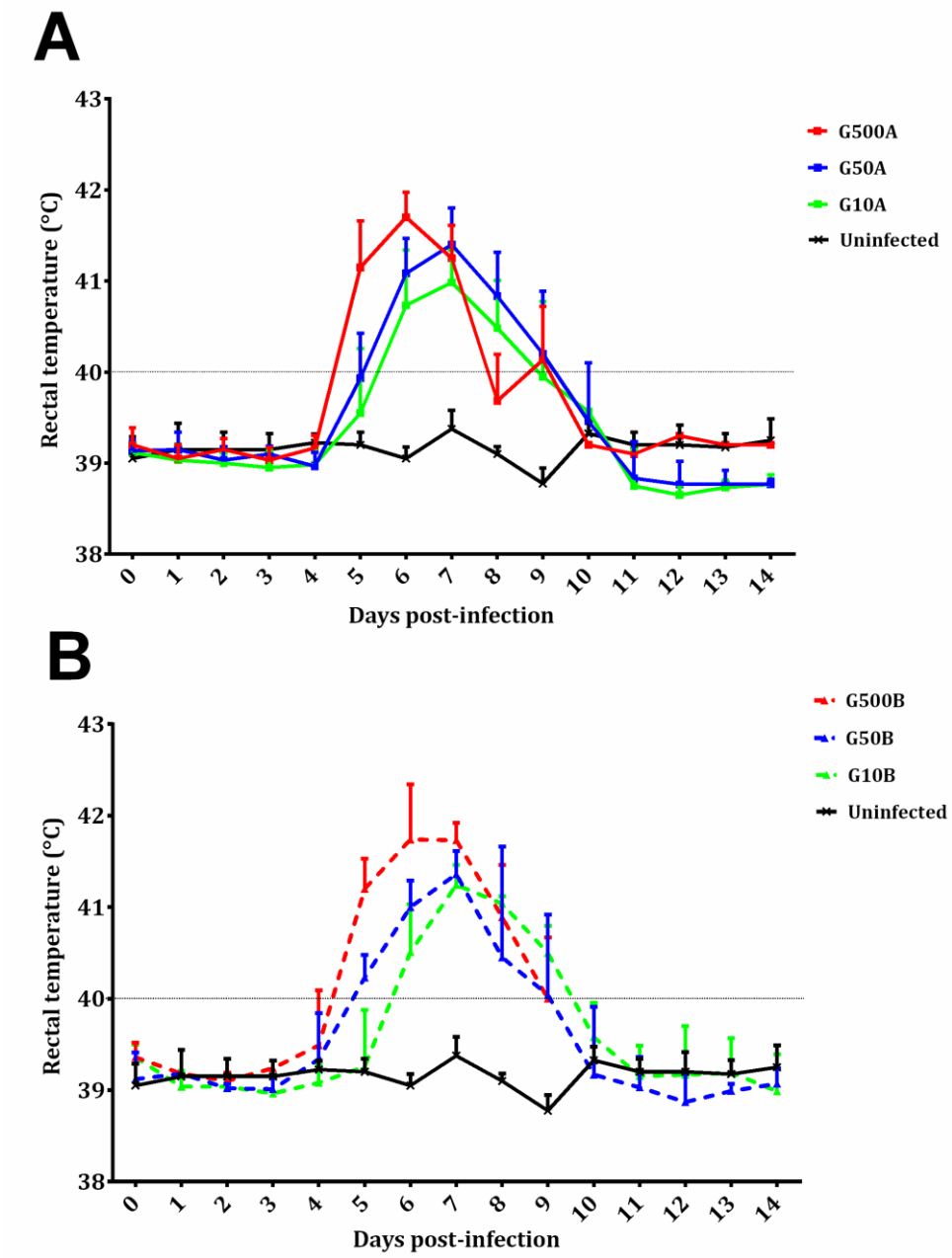
No mortality was found in any sheep during the experiment. All ewes showed fever after infection. Significant increases in body temperature were found for three-four days

Figure 3 - Effect of oral administration of TgShSp1 oocysts in the mouse pups. (A) Body weight progression of neonates born from dams infected on day 7 of pregnancy with 2000 (group A), 500 (group B), 100 (group C) or 25 (group D) TgShSp1 oocysts and the uninfected group (group E). Each point represents the average body weight of all animals per group. (B) Dot-plot graph of *T. gondii* load in brain from surviving pups. Each dot represents individual values of parasite burden (number of parasites per µg of DNA), and medians are represented as horizontal lines. (****) indicates $P < 0.0001$.



after day 4 p.i. in all infected groups. From day 14 pi until the end of the experiment, no changes were detected in the infected groups. Likewise, compared to groups infected with 500 oocysts, one day of delay in the increase of rectal temperature (from day 5 to 6 pi) was found in groups infected with 50 and 10 oocysts (Figure 4). Differences in temperature increase between groups receiving the same dose of oocysts of the different *T. gondii* isolates were

Figure 4 - Rectal temperatures of ewes infected with TgShSp1 oocysts and the uninfected ones (A) and ewes infected with TgME49 oocysts and the uninfected ones (B). From day 7 pi onwards, some ewes aborted and were euthanized, and their data are not available. Each point represents the mean + S.D. at the different sampling times for each group.



generally not found. As an exception, lower rectal temperatures were only found on day 8 pi in groups infected with 500 and 10 oocysts of TgShSp1 compared to groups receiving the same doses of TgME49 oocysts (G500A *vs* G10A and G500B *vs* G10B) ($P < 0.05$). The mean rectal temperature in the uninfected group remained below 39.5°C throughout the monitoring period.

Perinatal mortality in groups receiving 500 oocysts was 100%. In groups receiving 50 and 10 oocysts, 68% of fetuses/lambs in G50A (in 6/6 ewes) and 66% in G10A (in 4/6 ewes) died after infection with TgShSp1, and 42% (in 2/5 ewes) of them died in both groups after infection with TgME49. Perinatal mortality was increased with the oocyst doses. A lower perinatal survival rate was found in the group infected with 500 TgME49 oocysts compared

with that infected 50 oocysts (G500B *vs* G50B) ($P < 0.05$). In addition, a significantly lower perinatal mortality was found in those infected with 500 oocysts compared with those infected with 10 oocysts for both isolates (G500A and G500B compared to G10A and G10B, respectively) ($P < 0.05$). No differences in perinatal mortality were found between groups infected with 50 and 10 oocysts (G50A and G50B compared to G10A and G10B, respectively). Comparing groups that received the same dose of sporulated oocysts but different isolates, no significant differences were found in the perinatal survival rate. Concerning fetal death during pregnancy, all ewes challenged with 500 oocysts (G500A and G500B) aborted, and in the groups infected with lower doses, abortions were found in 3/6 and 2/5 pregnant ewes infected with 50 oocysts of TgShSp1 and TgME49, respectively, and in 1/6 and 1/5 pregnant ewes infected with 10 oocysts of TgShSp1 and TgME49, respectively (Table 2). Non-aborted dams gave birth between days 143 and 149 of pregnancy, except one ewe infected with 50 TgME49 oocysts, which gave birth prematurely on day 134. In G50A, 1 mummified fetus and 3 stillborn lambs were found, and in G50B, G10A and G10B, one, eight and one stillborn lambs were delivered, respectively. Concerning morbidity in lambs born alive, weakness was found in 5 out of 5 and 1 out of 4 in groups infected with 50 oocysts, G50A and G50B, respectively, and in 2 out of 5 and 0 out of 4 in those infected with 10 oocysts, G10A and G10B, respectively. Therefore, morbidity in lambs born alive from the group infected with 50 TgShSp1 oocysts, G50A, was significantly higher than the corresponding TgME49 group, G50B ($P < 0.05$), whereas no significant differences in the number of weak lambs were found between lambs from groups infected with 10 oocysts or between lambs from groups receiving different doses of both isolates. Dams from the pregnancy control group gave birth two stillborn lambs and six healthy lambs between days 147 and 152 of pregnancy.

3.5.2. Parasite detection and burden in placental and fetal tissues

3.5.2.1. Placental tissues

In ewes suffering early abortions, no *T. gondii* DNA was detected in placentomes in TgME49-infected animals (i.e., G500B, G50B and G10B), and in ewes infected with TgShSp1, parasite DNA was only detected in one ewe infected with 500 oocysts (G500A), which aborted on day 9 pi (one positive placentome sample out of 30), and in one ewe infected with 50 oocysts (G50A), which aborted on day 10 pi (one positive placentome samples out of 18). In contrast, all placentomes from ewes showing late abortion were PCR-positive. In ewes that delivered stillbirths or live lambs, all cotyledons from TgShSp1-infected animals were PCR-positive. Among those challenged with TgME49 oocysts, 100% of cotyledon samples were positive in the group infected with 500 oocysts (G50B), while 75% of cotyledon samples were positive in the group infected with 10 oocysts G10B (Table 2) (Table S2). Concerning parasite burden (measured as the number of tachyzoites per milligram of tissue) in cotyledons from ewes that delivered stillbirths or live lambs, no differences were found between groups infected with 50 and 10 oocysts in any of the isolates. However, comparing both *T. gondii* isolates, parasite loads in cotyledons from ewes that gave birth in groups infected with 50 and 10 oocysts of TgShSp1 (groups G50A and G10A), both were lower compared to those infected with TgME49 (G50B and G10B) ($P < 0.05$) (Figure 5A).

3.5.2.2. Fetal tissues

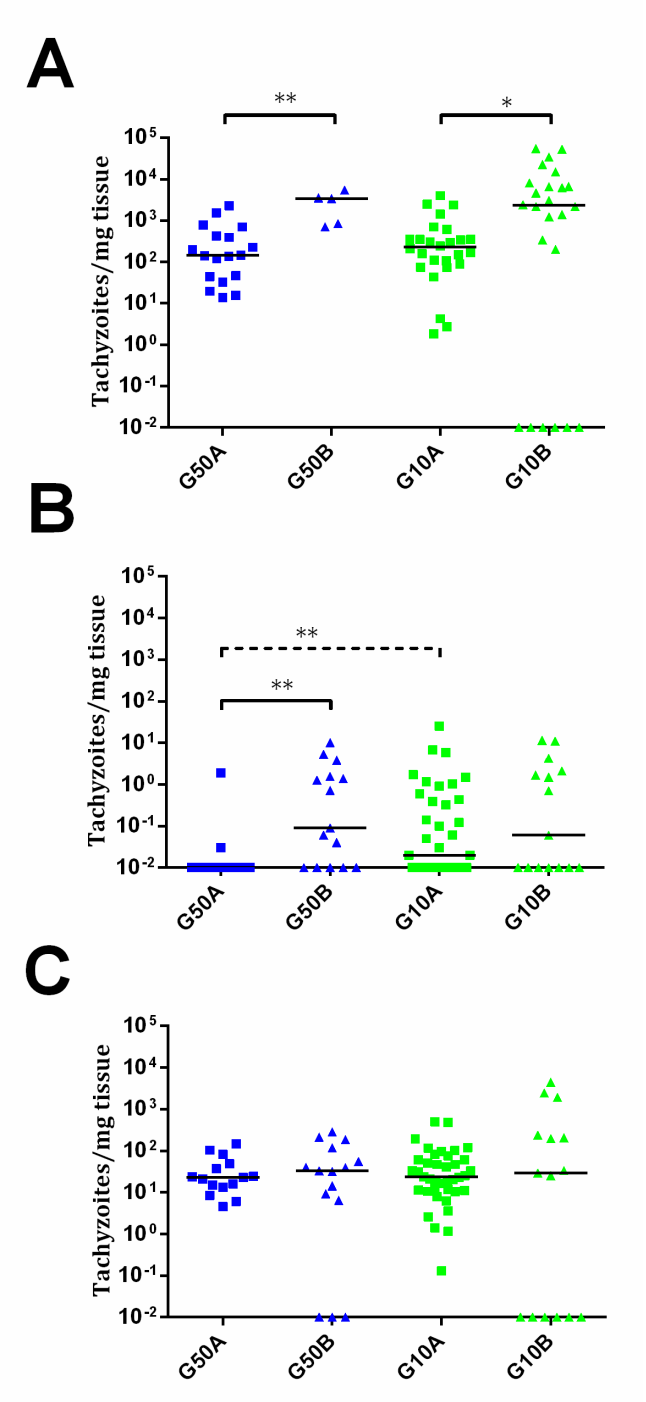
In tissues from fetuses undergoing early abortion upon TgME49 infection, no *T. gondii* DNA was detected. The same was true for fetuses from early abortions after challenge with TgShSp1 oocysts, except for one positive fetal lung sample (1 positive sample out of 20) from one ewe that aborted on day 10 pi in one

Table 2 - Perinatal mortality in sheep and percentages of placentomes/cotyledons or foetuses/lambs showing histological lesions and parasite detection.

Group	Perinatal mortality (%)	Clinical outcome	Number of ewes	Number of foetuses/lambs	Placentomes/cotyledons		Foetal brain		Foetal lung	
					H/E (%)	PCR (%)	H/E (%)	PCR (%)	H/E (%)	PCR (%)
Group 500A (500 TgShSp1 oocysts)	100	Early abortions	5	11	NA	16	100	-	-	-
		Late abortions	1	1	100	100	100	100	-	100
Group 500B (500 TgME49 oocysts)	100	Early abortions	5	10	NA	-	100	-		-
Group 50A (50 TgShSp1 oocysts)	68	Early abortions	3	7	NA	16	100	-	-	14
		Stillborns/lambs	3	9	NA	100	63	60	-	100
Group 50B (50 TgME49 oocysts)	42	Early abortions	1	1	NA	-	100	-	-	-
		Late abortions	1	1	100	100	100	100	-	100
		Stillborns/lambs	3	5	NA	100	100	100	-	100
Group 10A (10 TgShSp1 oocysts)	66	Late abortions	1	2	100	100	100	100	-	100
		Stillborns/lambs	5	13	NA	100	69	100	-	100
Group 10B (10 TgME49 oocysts)	42	Early abortions	1	2	NA	-	100	-	-	-
		Stillborns/lambs	4	5	NA	75	60	60	-	50

NA: not available

Figure 5 - Dot-plot graphs of *T. gondii* burdens in cotyledons from ewes that gave birth (A) and brain (B) and lung (C) from stillborn lambs and live lambs from *T. gondii*-infected ewes. Each dot represents individual values of parasite burden (number of parasites per milligram of host tissue), and medians are represented as horizontal lines. Considering that the *T. gondii* detection limit by real-time PCR is 0.1 parasites, negative samples (0 parasites) were represented on the log scale as <0.1 (i.e., 10^{-2}). The unbroken line is used to indicate differences between isolates, and the dashed line (----) is used to indicate differences between doses. For significant differences between infected groups in each tissue, (*) indicates $P < 0.05$ and (**) indicates $P < 0.01$.



group infected with 50 oocysts (G50A) (Table 2) (Table S2).

In late abortions, *T. gondii* DNA was detected in every fetus and all organs analyzed (Table 2). The percentage of positive samples in every individual organ ranged from 33% in the brain from one fetus in G10A to 100% in the rest of the brains and lungs from late abortions (Table S2).

In all stillbirths and live lambs, *T. gondii* DNA was found in at least one of the studied organs, except in one stillborn lamb and one live lamb born from one ewe infected with 10 TgME49 oocysts (G10B) in which *T. gondii* DNA was not detected in any analyzed tissue (Table S2). Concerning parasite detection in the brain of stillbirths/live lambs born from ewes infected with TgME49 oocysts, more samples were PCR-positive in the group infected with 50 oocysts, G50B (91.6%; 11/12; 4 out of 4 fetuses) compared to the group infected with 10 oocysts, G10B (53.3%; 8/15; 3 out of 5 fetuses) ($P < 0.05$). In lambs from ewes infected with TgShSp1 oocysts, a lower number of brain samples were found to be PCR-positive in the group infected with 50 oocysts, G50A (33.3%; 5/15; 7 out of 9 fetuses), compared to the group infected with 10 oocysts, G10A (66.6%; 26/39; 13 out of 13 fetuses) ($P < 0.05$). Similarly, lower parasite burden in brain from lambs was found in the group infected with 50 TgShSp1 oocysts (G50A *vs* G10A ($P < 0.01$). Comparing both isolates, parasite detection and parasite burden were higher in brain samples from lambs in the group infected with 50 oocysts of TgME49 compared to the corresponding TgShSp1 group G50B vs G50A) ($P < 0.01$), while no significant differences were found between groups infected with 10 oocysts (G10A *vs* G10B) (Table 2) (Figure 5B) (Table S2). In lung tissues from stillbirths/live lambs, all samples were PCR-positive in those groups infected with 50 oocysts (G50A and G50B). Additionally, 100% parasite detection was observed in the group infected with 10 oocysts of TgShSp1 (G10A), whereas a significantly lower parasite detection rate was found in the group infected with 10 oocysts of TgME49, G10B (60%; 9/15; 3 out of 6 animals) ($P < 0.05$). Comparison of the same oocyst dose from both isolates revealed no differences in parasite detection in lung samples from groups infected with 50 oocysts (G50B and G50A), but a higher parasite detection rate was found in the group infected with 10 TgShSp1 oocysts, G10A, compared to the group infected with 10 TgME49 oocysts, G10B ($P < 0.001$) (Table 2) (Table S2). No differences in parasite burden in lungs from lambs were found between different doses or isolates (Figure 5C). Likewise, no differences in parasite detection or parasite burden were found in any fetal tissue between stillborn lambs and live lambs (data not shown). Samples from fetal tissues exhibiting DNA degradation and mummification were excluded from PCR analysis.

3.5.3. Histological lesions and lesion quantification

The only evident histological lesions in the studied organs were found in the brain from fetuses/lambs and placenta. Only the placenta from late abortions detected through US was available for histological study. As in those cases of early abortions, lambing or delivery of stillbirths, it was too autolytic to allow proper histological evaluation.

In early abortions, multifocal areas of coagulative necrosis at the white matter (leukomalacia) were found in the brain from all the fetuses aborted in this period. In addition, no evident differences in the severity or number of lesions were noted between groups. In late abortions, lesions (multifocal necrotic placentitis) were found in all placentas studied. Likewise, there were brain lesions (multifocal nonpurulent encephalitis) in all the fetuses from late abortions (Table 2).

In stillbirths/live lambs, glial foci with or without a central area of necrosis were observed in the brain. These lesions were found in lambs from all groups, with a prevalence between 60% and 100%, depending on the group (Table 2). When the percentages of brain lesions in lambs from ewes infected with TgME49 and TgShSp1 were compared, there were no differences between isolates or oocyst doses tested. Furthermore, there was no difference in the percentage of cases with brain lesions between stillbirths and live lambs (data not shown). Lesion quantification was carried out in brain samples from the stillbirths and live lambs, and no significant difference was found in the number of lesions, individual focus area or percentage of damaged area between groups (Figure S4).

3.5.4. Humoral immune responses

The *Toxoplasma*-specific IgG antibody responses in dams are shown in Figure 6. No increase in IgG level compared to the uninfected group and no seroconversion was found in any of the ewes showing abortion during the acute phase of the infection. However, ewes with late abortion or those giving birth seroconverted on day 21 pi. In the group infected with 500 TgShSp1 oocysts, 500A, all ewes except one suffered early abortions, so this group was excluded from statistical analysis. From day 21 pi onwards, ewes infected with 50 and 10 oocysts seroconverted and exhibited higher IgG compared to the control group ($P < 0.05$). When analyzing the IgG levels of animals infected with TgShSp1 sporulated oocysts, no significant differences were found between ewes infected with 50 and 10 oocysts, G50A and G10A (Figure 6A). However, in animals infected with TgME49 sporulated oocysts, it is noteworthy that the group infected with 10 oocysts, G10B, had higher IgG on day 21 pi than the group infected with 50 oocysts, G50B ($P < 0.01$) (Figure 6B). Comparing groups receiving the same dose of sporulated oocysts, no significant differences in IgG level were found between groups infected with 50 oocysts, G50A and G50B. However, the group infected with 10 oocysts of TgShSp1, G10A, displayed lower IgG than that infected with TgME49, G10B, from day days 21 to 35 pi ($P < 0.01$). All uninfected control animals exhibited basal IgG levels within the reference range throughout the experimental study.

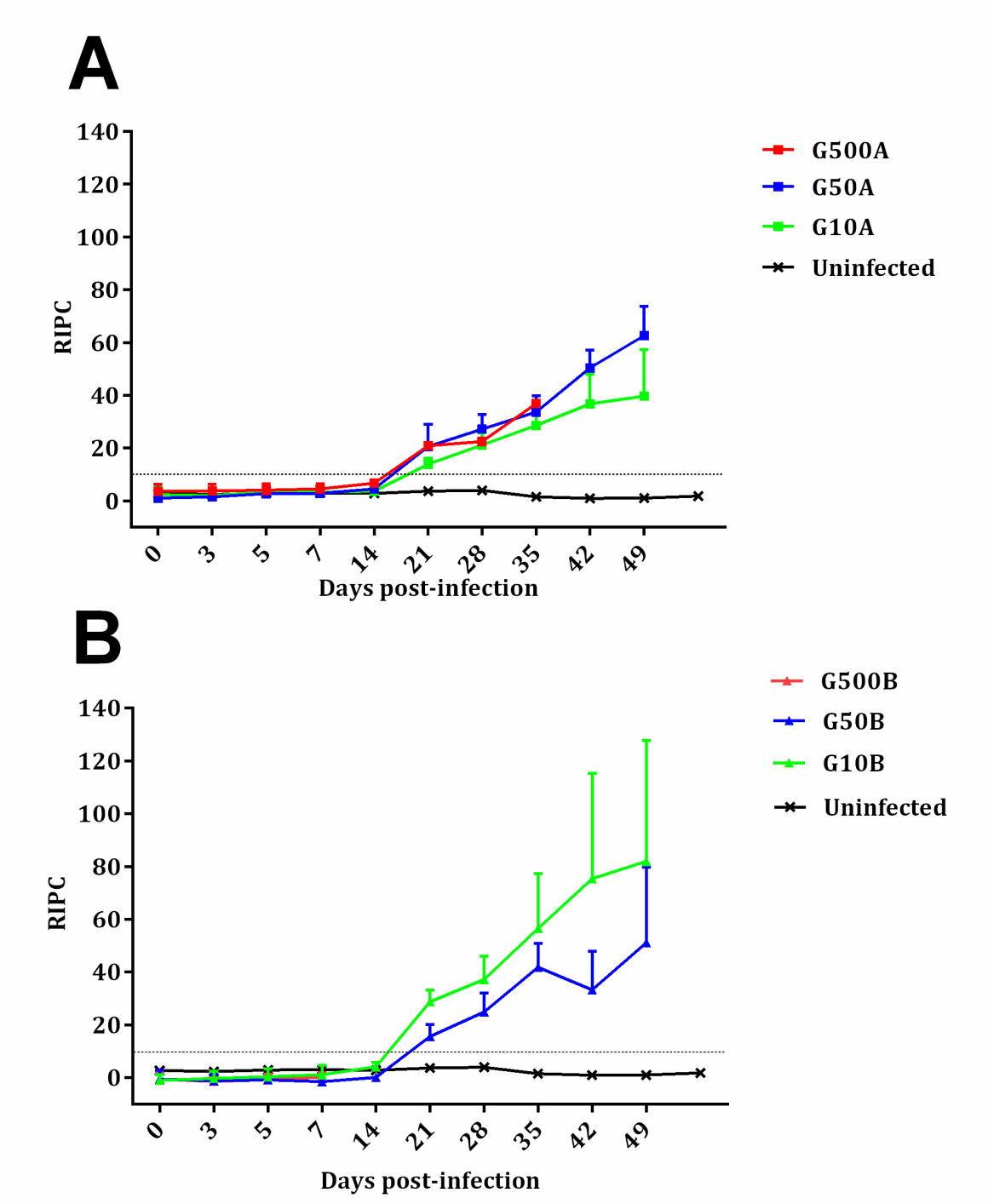
None of the fetuses that were aborted before day 11 pi had detectable IgG against *T. gondii* antigen. In contrast, fetuses undergoing late abortions were IgG-positive. Of the lambs born from TgShSp1-infected ewes, seven out of eight and nine out of twelve lambs were positive in groups infected with 50 and 10 TgShSp1 oocysts, G50A and G10A, respectively. Similarly, of the lambs born from TgME49-infected ewes, two out of three and 50% of lambs born from those groups infected with 50 and 10 TgME49 oocysts, G50B and G10B, respectively, were positive (Table S3). Specific IgG responses against parasite antigen were not detected in lambs from the uninfected group.

3.6. Comparative assessment of congenital infection in mice and sheep after infection with TgShSp1 oocysts.

Perinatal mortality after infection with 500 TgShSp1 oocysts occurred in sheep at a statistically higher rate compared to mice ($P < 0.001$), since in sheep all fetuses died and in mice only 50% of the pups died. Similarly, higher perinatal mortality was found in sheep compared to mice after infections with intermediate ($P < 0.0001$) and low doses of oocysts ($P < 0.01$), since infection with 50 and 10 TgShSp1 oocysts triggered mortality in 68 and 66% of fetuses/lambs, whereas in mice mortality of 9% and 27% of the pups was observed after infection with 100 and 25 TgShSp1 oocysts, respectively.

Since no lambs were born in the group infected with 500 TgShSp1 oocysts, offspring morbidity in this group could not be assessed.

Figure 6 - Kinetics of the antibody production in ewes infected with TgShSp1 oocysts and the uninfected ones (A) and ewes infected with TgShSp1 oocysts and the uninfected ones (B). From day 7 pi onwards, some ewes aborted and were euthanized, and their data are not available. Each point represents the mean + S.D. at the different sampling times for each group. Serum levels of total IgG antibodies against *T. gondii* are expressed as a relative index percent (RIPC), according to the formula: RIPC = (OD405 sample – OD405 negative control)/(OD405 positive control – OD405 negative control) × 100.



However, in mice infected with 500 TgShSp1 oocysts, a decrease in pup body weight was found from day 14 pi onwards. Offspring from mice infected with 100 TgShSp1 oocysts showed a decrease in bodyweight from day 14 pi onwards, and all lambs born alive from ewes infected with 50 TgShSp1 exhibited weakness at birth. At low doses of oocysts, no body weight decrease was noted in pups born from mice infected with 25 TgShSp1 oocysts; however, 2 out 5 live lambs from ewes infected with 10 TgShSp1 oocysts were born with obvious weakness and impaired health.

All (100%) of the surviving mouse pups were PCR-positive in the brain, and 100% live lambs were seropositive or with a PCR-positive result in at least one tissue after infection with intermediate doses of oocysts (100 TgShSp1 oocysts in mice and 50 TgShSp1 oocysts in sheep). Additionally, 100% of the surviving pups were PCR-positive in the brain after infection with 500 TgShSp1 oocysts (high dose of oocysts). However, a statistically higher number of *T. gondii*-positive offspring were found in sheep compared to mice after infection with low doses of oocysts ($P < 0.05$), since 100% lambs were seropositive or with a PCR-positive result in at least one tissue after infection with 10 TgShSp1 oocysts and only 37% of the surviving pups were PCR-positive in the brain after infection with 25 TgShSp1 oocysts (Table 3).

4. Discussion

T. gondii is an apicomplexan parasite that is distributed worldwide (Dubey, 2010). In Europe and North America, *T. gondii* isolates display a clonal population structure, with the vast majority of *T. gondii* isolates being grouped into three lineages, namely, types I, II and III (Howe and Sibley, 1995). Type II *T. gondii* is the most prevalent in all hosts in Europe, including sheep (Dumètre *et al.*, 2006; Halos *et al.*, 2010a; Su *et al.*, 2010). Previous studies in Europe have shown that *T. gondii* type II is associated with ovine abortion (Owen and Trees, 1999; Jungersen *et al.*, 2002; Chessa *et al.*, 2014). In Spain, type II is the most prevalent genotype in wild animals and cats (Montoya *et al.*, 2008; Calero-Bernal *et al.*, 2015), as well as in previously obtained ovine isolates (Fuentes, 1999). The TgShSp1 isolate belongs to genotype #3 (a type II variant, II for nine alleles/I for Apico), sharing genotype with the Prugniaud (PRU) isolate.

T. gondii PRU isolates exhibit a similar genetic pattern to the *T. gondii* type II reference isolate, TgME49 (genotype #1) (Su *et al.*, 2012). In addition, both type II isolates, TgME49 and PRU, activate the host cell transcription factor NF-κB, an integral component of the immune response to *T. gondii*, and they display identical GRA15 gene sequences, which is involved in NF-κB activation (Rosowski *et al.*, 2011). TgME49 was isolated from sheep muscle in 1958 (Lunde and Jacobs, 1983) and has since then undergone long-term passaging in cell culture and mice (Sibley *et al.*, 2002). Previous studies have demonstrated changes in biological characteristics of *T. gondii* isolates after passages in mice and cell culture (Frenkel *et al.*, 1976; Lindsay *et al.*, 1991; Harmer *et al.*, 1996; Saraf *et al.*, 2017). This fact has been widely studied in *T. gondii* type I isolates (Cesbron and Sabin, 1994; Villard *et al.*, 1997; Dubey *et al.*, 1999; Mavin *et al.*, 2004; Khan *et al.*, 2009b). However, whether these changes also occur in type II isolates, and how they compare to recently obtained isolates, remains unknown. Increased growth *in vitro* can be found after repeated passages (Yano *et al.*, 1987). The dramatic differences observed during *in vitro* growth of TgME49 and TgShSp1 might reflect the highly different passage history of the two isolates. Plaque formation is commonly used to measure growth of *T. gondii*, and this process is the result of several events, including invasion, growth, egress, and migration (Roos *et al.*, 1995). Notably, TgME49 tachyzoites formed plaques at 4 days pi, but TgShSp1 did not. The finding that TgShSp1 did not form

Table 3 - Summary of the outcome of the congenital infection in mice and sheep after infection with different doses of TgShSp1 oocysts.

Doses of oocysts (Mice/Sheep)	Perinatal mortality[a] (Mice/Sheep)	Morbidity in the offspring[b] (Mice/Sheep)	Vertical transmission[c] (Mice/Sheep)
High (500 oocysts/500 oocysts)	Medium/High***	High/NA	High/NA
Intermediate (100 oocysts/50 oocysts)	Low/High****	High/High	High/High
Low (25 oocysts/10 oocysts)	Low/Medium**	-/Medium	Medium/High*

[a] Mortality of foetuses during pregnancy in sheep and of pups/lambs after birth in mice and sheep.
[b] Morbidity in the offspring was evaluated by decrease of the body weight of pups from day 14 pp in mice and by clinical signs in live lambs (weakness) at birth.
[c] In mice, PCR-positive brains in surviving pups at day 28 pp. In sheep, seropositive live lambs with a PCR-positive result in at least one tissue.
NA: not available
High, medium, low or minus (-) mean presence of clinical signs, perinatal mortality or vertical transmission of the parasite in >67%, 66-34%, <33% and 0% of the foetuses/pups/lambs, respectively.
Body weights lower than the uninfected group are considered high morbidity, while no difference compared to uninfected group is represented as minus (-).
(*) indicates $P < 0.05$, (**) indicates $P < 0.01$, (***) indicates $P < 0.001$ and (****) indicates $P < 0.0001$ significant differences between mice and sheep in the number of foetuses/pups/lambs died or in the number of surviving offspring infected with *T. gondii*.

plaques *in vitro* could have resulted from the limited growth rate, associated with a higher capacity of bradyzoite conversion, as previously described Khan *et al.* (2009b). This was confirmed by monitoring spontaneous cyst formation through labeling with the fluorescent lectin DBL, demonstrating tissue cyst formation under standard cell culture procedures in TgShSp1 that was greater than TgME49. Due to its high passage number in cell culture or mice, our TgME49 isolate may not accurately represent natural virulence traits of the type II lineage, which suggests that comparisons of phenotypes between *T. gondii* isolates should be conducted using low-passage stocks.

Traditionally, mouse models are utilized to evaluate virulence by monitoring survival after experimental infection. Type I isolates are highly virulent in mice (LD_{100} of 1 tachyzoite), whereas types II and III exhibit median lethal doses (LD_{50}) that range from 10^2 to 10^5 (Saeij *et al.*, 2006). Conventionally, TgME49 is a cystogenic type II isolate with low virulence in mice (Ferreira *et al.*, 2001; Gavrilescu and Denkers, 2001; Oliveira *et al.*, 2016). Intraperitoneal inoculation of 10^3 and 5 x 10^4 TgME49 tachyzoites intraperitoneally has not caused mortality (Ferreira *et al.*, 2001; Oliveira *et al.*, 2016). However, in this study, TgME49 displayed a LD_{50} of 10^3, so the virulence of our TgME49 could be considered stronger than previous descriptions (Ferreira *et al.*, 2001; Oliveira *et al.*, 2016). Enhanced virulence in mice for *T. gondii* strains maintained for several passages has also been reported previously (Shimizu *et al.*, 1967; Sibley and Boothroyd, 1992; Frenkel and Ambroise-Thomas, 1996). Hence, it seems logical to speculate that results from studies using laboratory isolates should be validated with more recent isolates before they can be extrapolated as general features of the respective lineage. In contrast to TgME49, mice inoculated with tachyzoites of the recently obtained type II isolate TgShSp1 exhibited only moderate, low-level clinical signs, but no mortality ($LD_{50} > 10^5$), similar to what was described earlier after intraperitoneal inoculation of Swiss Webster mice with 10^3 tachyzoites of a PRU isolate (Wang *et al.*, 2013). In addition, although oocysts are considered more virulent than tachyzoites in mice (Dubey and Frenkel, 1973), no mortality in adult mice was found after infection with oocysts of TgShSp1 in pregnant and non-pregnant mice, suggesting very low virulence in mice.

In the present work, we also investigated congenital toxoplasmosis in pregnant mice by inoculating them orally with different doses of TgShSp1 oocysts. The risk of congenital toxoplasmosis depends on the virulence of the parasite (Tenter *et al.*, 2000). Based on the previously established toxoplasmosis model using TgME49 oocysts (Müller *et al.*, 2017c), we infected mice at day 7 postmating, which represents the beginning of the second term of gestation. Few mice infected with TgShSp1 oocysts showed mild clinical signs, contrary to the large number of mice succumbing to infection after the same oocyst doses of TgME49 (Müller *et al.*, 2017c). There is a possibility that there has been a selection towards increased virulence within TgME49 due to the successive passages, as explained above but also due to the sulfadimidine treatment that was applied in mice used for infection of cats to generate TgME49 oocysts used in this study (Müller *et al.*, 2017c). This sulfonamide treatment could act as a bottleneck in selecting tachyzoites with a faster replication and therefore increasing the virulence in mice of the final TgME49 parasites. Unlike what was observed for TgME49 (Müller *et al.*, 2017c), with which a clear effect on pregnancy rate was found after infection with 2000 oocysts and on litter size after infection with 500 oocysts, infection with TgShSp1 oocysts did not result in alteration of pregnancy rate or litter size. Likewise, while 92% of the pups died after infection of dams with 25 TgME49 oocysts (Müller *et al.*, 2017c), a significant decrease in pup survival was found only after infection

with 2000 and 500 TgShSp1 oocysts, where there was mortality in 50% of the pups. It has been proposed that immune modulation in pregnant mice (Athanassakis and Iconomidou, 1996) generally renders them more susceptible to infection, but in this study, infection with TgShSp1 oocysts did not show differences in cerebral or lung infection between pregnant and non-pregnant mice. In conclusion, infection of mice with TgShSp1 oocysts at mid-pregnancy did not generate severe clinical signs in adult mice, but infection doses of 2000 and 500 oocysts in the dams resulted in mortality of 50% of the pups and decreased body weight in surviving pups, and 100% vertical transmission occurred with doses of up to 100 oocysts.

Sheep are a relevant host of the parasite and could suffer abortions when primo infected during gestation (Vargas-Villavicencio *et al.*, 2016). In this study, although rectal temperatures in sheep were similarly increased in TgME49 and TgShSp1, ewes infected with 500 and 10 TgME49 oocysts exhibited higher rectal temperature on day 8 pi compared to same doses of TgShSp1 oocysts. Perinatal mortality was similar for both isolates. However, those ewes infected with 10 and 50 TgME49 oocysts and that delivered stillbirths/live lambs exhibited higher parasite load in cotyledons than those infected with the same doses of TgShSp1 oocysts. Similarly, a higher parasite load was found in the brain from lambs born in the group infected with 50 TgME49 oocysts compared to the corresponding TgShSp1 group. Therefore, it is tempting to hypothesize that the enhanced virulence of our TgME49 contributed to the abovementioned effects. Comparing different doses of infection, pregnant ewes challenged with 50 and 10 oocysts showed one day of delay in the increase of rectal temperature compared to ewes infected with 500 oocysts in both isolates, similar to what was described by Buxton *et al.* (1991) and Mévélec *et al.* (2010). Likewise, there is a correlation between the dose of infection and the rate of early abortions, as previously suggested (Mévélec *et al.*, 2010; Benavides *et al.*, 2017). Infection with 500 oocysts triggered abortion in all fetuses, similar to previous experimental infections in pregnant sheep at mid-pregnancy using 2000 oocysts (Owen *et al.*, 1998a; Castaño *et al.*, 2014). After infection with 50 TgShSp1 oocysts or 50 TgME49 oocysts, 68 and 42% of fetuses/lambs died, respectively, similar to what was previously reported after infection with 50 M4 oocysts (Castaño *et al.*, 2014). The occurrence of abortions after infection with 10 oocysts was low, but large numbers of stillbirths and weak lambs were found, mainly in the 10 TgShSp1 oocysts group. There seems to be a correlation between the presence of the parasite and the occurrence of stillbirths, since stillbirths from the group infected with 10 TgShSp1 oocysts exhibited higher parasite detection and load in the brain than those in the group infected with 50 TgShSp1 oocysts. Regardless of the isolate or dose, no differences were found in the congenital infection of lambs born, since vertical transmission was found in all them except in one stillborn lamb and one live lamb born from one ewe infected with 10 TgME49 oocysts. Likewise, no differences were found between doses or isolates with respect to brain lesion presence and lesion severity in lambs born.

Most of the experimental studies in pregnant sheep carried out so far used M1, M3 and M4 *T. gondii* type II isolates for infection (Dubey, 2009b; Castaño *et al.*, 2014). However, although numerous experimental infections were also carried out in mice with these isolates (Nicoll *et al.*, 1997; Owen *et al.*, 1998a; Hamilton *et al.*, 2018), their virulence in mice models has not been studied in depth. Therefore, the correlation between virulence in mice and outcome of experimental infections in pregnant sheep has not been elucidated. Despite the clear differences in body weight between mice and sheep, in the current study, similar doses of oocysts were used to compare both hosts. None of the adult mice challenged with 25 TgShSp1 oocysts exhibited clinical signs, whereas all ewes challenged with 10

TgShSp1 oocysts had fever. Therefore, morbidity in sheep seems to be higher than in mice. In addition, no mortality was observed in adult mice or sheep infected with TgShSp1 oocysts. When comparing the congenital infection after challenge at mid-pregnancy between both hosts, 50% mortality was caused in mice by infection with 500 TgShSp1 oocysts, whereas in sheep infection with the same oocyst dose caused mortality in all fetuses. In brief, mice seem to be less susceptible to perinatal mortality than sheep, despite the fact that vertical transmission was similar in both species. High vertical transmission and low offspring mortality could be an evolutionary strategy of the parasite to generate a large infected offspring group in mice, one of the most relevant hosts of *T. gondii* (Müller and Howard, 2016).

There are several differences between mice and sheep that could underlie the differences found in this study. The histological structure of the placenta is very different between mice and sheep (Entrican, 2002), and although maternal blood and fetal tissue are closer in mice, allowing an easy crossing of tachyzoites but also of antibodies, the longer period of gestation, the lack of maternal antibodies crossing the placental barrier and fewer fetuses may facilitate vertical transmission in sheep. In addition, host genetics are likely important in determining susceptibility and severity of infection (Howe *et al.*, 1996; Müller and Howard, 2016). Small rodents, natural intermediate hosts, are often exposed to a higher dose and more virulent parasites than other groups of mammals. It may therefore be that Toll-Like-Receptors (TLR)11 and TLR12 and the polymorphism of immunity-related GTPases (IRG proteins) have been positively selected in rodents, because of their critical importance in host resistance against high infection loads or more virulent clones of *T. gondii*. In mice, TLR11 and TLR12 on dendritic cells detect the apicomplexan actin-binding protein profilin leading to the secretion of interleukin 12 (IL12), which can subsequently induce production of IFNγ by T cells. IFNγ induces a variety of parasiticidal mechanisms, which in mice are dominated by upregulation of the IRGs. IRGs can destroy the vacuole in these parasites live and subsequently the parasite itself (Gazzinelli *et al.*, 2014). Considering the ubiquity of *T. gondii* in nature, it is intriguing that genes encoding TLR11, TLR12, and IRG proteins are not found in many mammalian species (Gazzinelli *et al.*, 2014). Although further studies are needed, and despite the influence of genetic polymorphisms in ovine abortions (Darlay *et al.*, 2011), this fact could render sheep less resistant. Similarly, differences in immune cell populations may influence the pathogenesis of toxoplasmosis in these hosts. γδ T cells, which rapidly recognize and respond to nonprocessed antigens and seem to have an important role in *T. gondii* infection (Egan *et al.*, 2005), represent a relevant subset of circulating T cells in sheep compared to mice (Holderness *et al.*, 2013). Further studies are needed to characterize the cellular and molecular bases contributing to transmission dynamics and disease in different hosts of *T. gondii* (Dubremetz and Lebrun, 2012; Hunter and Sibley, 2012).

In conclusion, we have demonstrated that infection with tachyzoites and oocysts of the type II *T. gondii* isolate TgShSp1 in mice does not cause mortality, but this isolate is efficiently vertically transmitted in pregnant mice, and compared to sheep, it triggers lower offspring mortality and morbidity. Thus, at least for this isolate, the disease caused in pregnant mice and offspring is not a reliable predictor/indicator for disease caused in pregnant sheep at mid-gestation. Whether this conclusion is also valid for other type II *T. gondii* strains needs to be addressed in future studies. In addition, our results suggest that the laboratory isolate TgME49 exhibits an enhanced virulence due to successive passages in cell culture and mice. Thus, virulence traits may have been modified, and it might be advisable to use low-passage isolates in experimental studies, as these probably provide

a more realistic picture of the true nature of the parasite biology in the field.

Acknowledgments

We thank Dr. J.C. Boothroyd for the donation of the TgME49 isolate. We gratefully acknowledge Norbert Müller, Pablo Winzer and Nicoleta Anghel and Vreni Balmer from the University of Bern (Switzerland), Melchor Molero Crespo, Iván Panero Frade, Carmen Espiniella García and María Jesus González Fraile from IGM (León, Spain), Alejandro Jiménez, Cristina Guerrero, Alicia Colos and Irene Écija from SALUVET group, Müller Ribeiro from University of Pernambuco (Recife, Brazil) and Jose Calasanz Jiménez, Francisco Saura and Jose Maria González from University of Zaragoza (Zaragoza, Spain) for their excellent technical assistance. The Animal Experimentation Service (SEA) at the University of Zaragoza is acknowledged for providing their facilities to carry out the reproductive program in sheep. Roberto Sánchez Sánchez is supported by a fellowship from the Spanish Ministry of Education, Culture and Sports (MECD), as a part of the Program of Training of University Teaching Staff (FPU, grant number FPU13/03438) and a mobility grant for predoctoral short stays in R+D centers (EST16/0719). Daniel Gutierrez Expósito is the recipient of a postdoctoral contract from the "Junta de Castilla y León", partially funded by the European Social Fund (European Union). Noive Arteche is the recipient of a predoctoral contract from the Ministerio de Economía, Industria y Competitividad (Ref. BES-2016-076513). This work was supported by the Ministry of Economy and Competitiveness (AGL2016-75935-C2-1-R and C2-2-R), the Community of Madrid, Spain (PLATESA, S2013/ABI2906), Junta de Castilla y León (LE080U16) and a grant of the Swiss National Science Foundation to AH (project No. 310030_165782).

Author contributions

IF, JR, VP, AH, LMO and JB conceived the study and participated in its design. LMO coordinated the isolation, *in vitro* and mouse studies, and JB and LMO coordinated the studies in sheep. RS, LMO and JB wrote the manuscript, with result interpretation and discussion inputs from IF, JR and AH. JR, JM and JB carried out the isolation of TgShSp1. RS and JR carried out *in vitro* experiments. LMF selected sheep and executed the reproductive program. RS, IF, JR and JM prepared tachyzoites or oocysts and performed the infections. RS, DG, NA, JM, AA, VP and JB participated in inoculation and clinical examination of animals, performed necropsies and sampling of the animals and performed histopathological analyses. RS performed PCR and qPCR analyses, serological assays, and statistical analysis and interpreted the results. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Table S1 - Clinical signs, serological titres and parasite detection in mice intraperitoneally infected with TgME49 and TgShSp1 tachyzoites.

Dose of tachyzoites	Mice ref.	Clinical signs (dpi)*	IFAT titre	PCR	
				Brain	Lung
10^5 TgShSp1	**1**	1 (4)	1:400	+	-
	2	1 (5)	1:800	+	+
	3	1 (7)	1:1600	+	+
	4	1 (9)	1:800	+	+
	5	1 (10)	1:1600	+	+
10^5 TgME49	**1**	3 (11)^T	Negative	+	+
	2	3 (8)^T	NA	+	+
	3	2 (8)	1:800	+	+
	4	2 (8)	1:800	+	+
	5	2 (8)	1:400	+	+
10^4 TgShSp1	**1**	2 (10)	1:800	+	+
	2	1 (8)	1:800	+	+
	3	1 (8)	1:1600	+	+
	4	1 (9)	1:800	+	+
	5	0	1:400	+	-
10^4 TgME49	**1**	3 (10)^T	Negative	+	+
	2	2 (9)	1:200	+	-
	3	3 (14)^T	Negative	+	+
	4	2 (9)	1:200	+	+
	5	2 (9)	1:800	+	-
10^3 TgShSp1	**1**	1 (6)	1:800	+	-
	2	1 (6)	1:400	+	+
	3	1 (7)	1:400	+	+
	4	1 (9)	1:1600	+	+
	5	1 (9)	1:800	+	-
10^3 TgME49	**1**	4 (33)^T	1:25	+	+
	2	4 (13)^T	Negative	+	+
	3	2 (10)	1:400	+	+
	4	2 (10)	1:400	+	-
	5	3 (14)^T	NA	+	+
10^2 TgShSp1	**1**	1 (7)	1:800	+	-
	2	1 (8)	1:1600	+	+
	3	1 (8)	1:1600	+	+
	4	1 (9)	1:3200	+	+
	5	1 (13)	1:800	+	+
10^2 TgME49	**1**	2 (14)	1:200	+	+
	2	4 (13)^T	Negative	+	+
	3	2 (14)	1:100	+	+
	4	2 (14)	1:100	+	+
	5	2 (14)	1:200	+	-

Table S1 – Continued.

Dose of tachyzoites	Mice ref.	Clinical signs (dpi)*	IFAT titre	PCR	
				Brain	Lung
10 TgShSp1	**1**	1 (7)	1:800	+	+
	2	0	1:800	+	+
	3	0	1:800	+	+
	4	0	1:800	+	+
	5	0	1:1600	+	+
10 TgME49	**1**	2 (14)	1:100	+	-
	2	2 (14)	1:100	+	-
	3	4 (24)[T]	Negative	+	+
	4	2 (14)	1:100	+	-
	5	2 (14)	1:50	+	-
1 TgShSp1	**1**	0	Negative	-	-
	2	0	Negative	-	-
	3	0	Negative	-	-
	4	0	Negative	-	-
	5	0	Negative	-	-
1 TgME49	**1**	0	Negative	-	-
	2	0	Negative	-	-
	3	0	Negative	-	-
	4	0	Negative	-	-
	5	0	Negative	-	-

* Clinical signs compatible with toxoplasmosis. Scores of 0 (no alterations), 1 (ruffled coat), 2 (rounded back), 3 (noticeable loss of body condition/severe weight loss) or 4 (nervous signs such as activity decrease, hind limb paralysis, walking in circles or head tilt). In brackets, day post infection when mice exhibited clinical signs.

[T] Mice showing clinical scores of 3 or 4 were euthanized to limit unnecessary suffering.

NA: not available

Figure S1 - Box-plots showing IgG serum titers generated in dams (A) and non-pregnant mice (B) infected with TgShSp1 oocysts and the uninfected group. Graphs represent the median percentage, the lower and upper quartiles (boxes) and minimum and maximum values (whiskers).

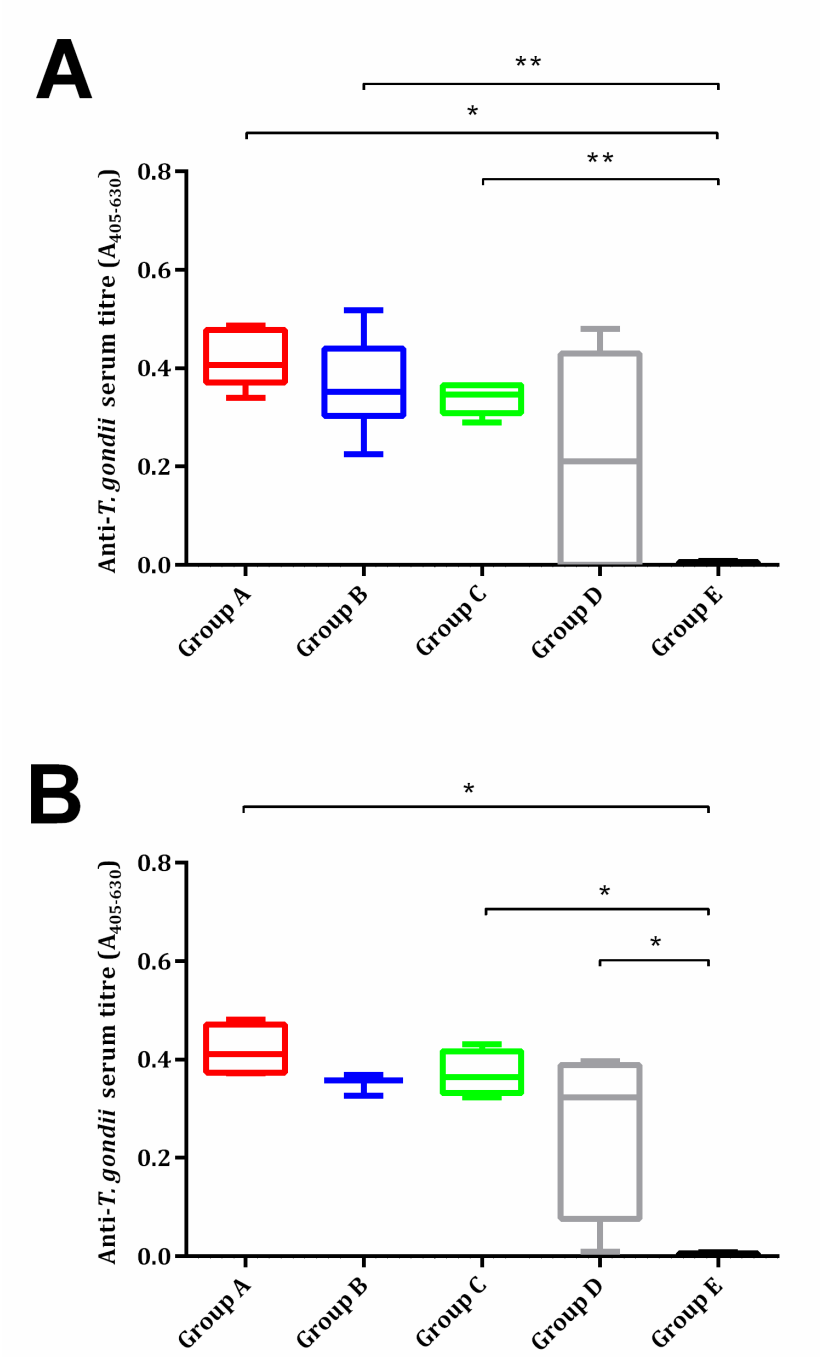


Figure S2 - Dot-plot graphs of *T. gondii* burdens in brain from dams (A) and non-pregnant mice (B). Each dot represents individual values of parasite burden (number of parasites per microgram of DNA), and medians are represented as horizontal lines.

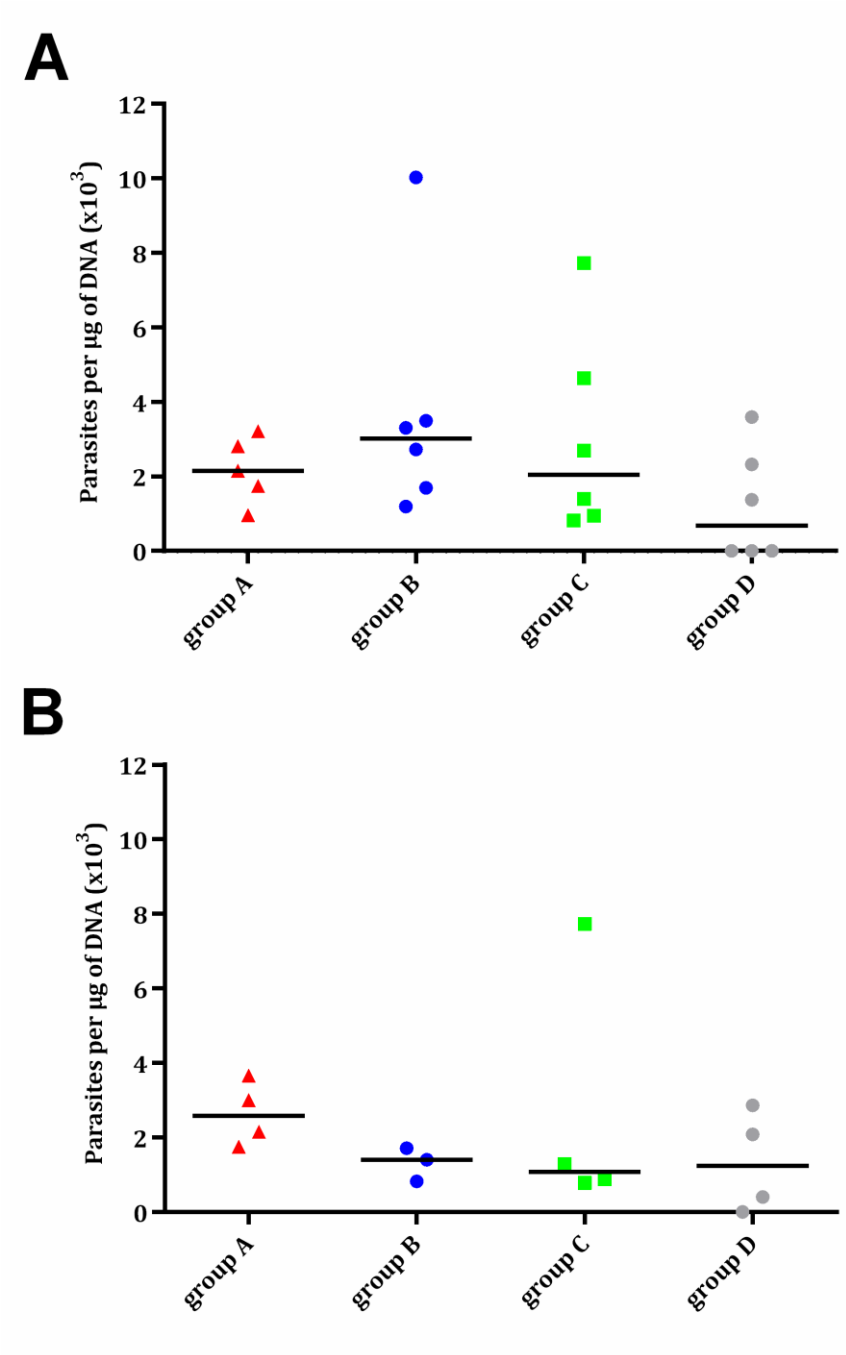


Figure S3 - Dot-plot graphs of *T. gondii* burdens in lung from dams (A) and non-pregnant mice (B). Each dot represents individual values of parasite burden (number of parasites per microgram of DNA), and medians are represented as horizontal lines.

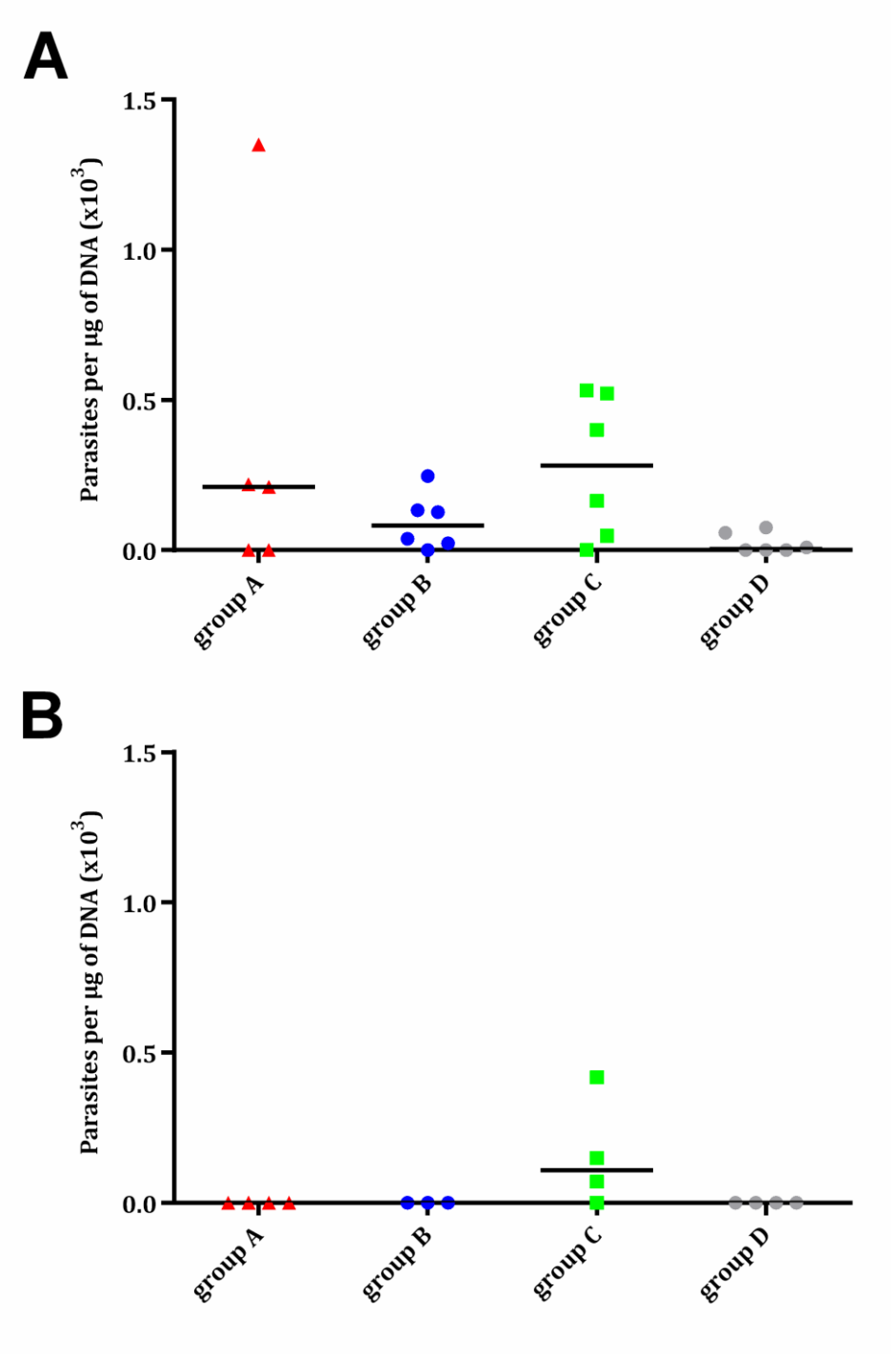


Table S2 - Individual frequency of parasite DNA detection in infected animals.

Group	Ewe ref.	Foetal death (dpi)[a]	Placentomes/ cotyledons[b]	Foetal/ lamb ref	Foetal tissues	
					Brain	Lung
G500A (500 TgShSp1 oocysts)	**500A.1**	9	+	500A.1F1	-	-
	500A.2	36	+++	500A.2F1	+++	+++
	500A.3	8	-	500A.3F1	-	-
	500A.4	9	-	500A.4F1	-	-
				500A.4F2	-	-
				500A.4F3	-	-
	500A.5	8	-	500A.5F1	-	-
	500A.6	8	-	500A.6F1	-	-
				500A.6F2	-	-
				500A.6F3	-	-
				500A.6F4	-	-
				500A.6F5	-	-
G500B (500 TgME49 oocysts)	**500B.1**	8	-	500B.1F1	-	-
				500B.1F2	-	-
	500B.2	9	-	500B.2F1	-	-
				500B.2F2	-	-
				500B.2F3	-	-
	500B.3	8	-	500B.3F1	-	-
				500B.3F2	-	-
	500B.4	9	-	500B.4F1	-	-
	500B.5	9	-	500B.5F1	-	-
				500B.5F2	-	-
G50A (50 TgShSp1 oocysts)	**50A.1**	a	+++	50A.1F1	-	+++
				50A.1F2	γ	γ
				50A.1F3	β	β
	50A.2	10	+	50A.2F1	-	+
				50A.2F2	-	-
				50A.2F3	-	-
	50A.3	9	-	50A.3F1	-	-
				50A.3F2	-	-
	50A.4	a	+++	50A.4F1	++	+++
				50A.4F2	++	+++
	50A.5	a		50A.4F3	γ	γ
			+++	50A.5F1	-	+++
				50A.5F2	+	+++
	50A.6	10	-	50A.6F1	-	-
				50A.6F2	-	-
G50B (50 TgME49 oocysts)	**50B.1**	35	+++	50B.1F1	+++	+++
	50B.2	a	NA	50B.2F1	+++	+++
	50B.3	11	-	50B.3F1	-	-
	50B.4	a	+++	50B.4F1	+++	+++
				50B.4F2	+++	+++
	50B.5	a	+++	50B.5F1	++	+++

Table S2 – Continued.

Group	Ewe ref.	Foetal death (dpi)[a]	Placentomes/ cotyledons[b]	Foetal/ lamb ref	Foetal tissues	
					Brain	Lung
G10A (10 TgShSp1 oocysts)	**10A.1**	49	+++	10A.1F1	γ	γ
				10A.1F2	+	+++
	10A.2	a	+++	10A.2F1	++	+++
				10A.2F2	+	+++
	10A.3	a	+++	10A.3F1	+++	+++
				10A.3F2	++	+++
				10A.3F3	++	+++
	10A.4	a	+++	10A.4F1	+++	+++
				10A.4F2	++	+++
	10A.5	a	+++	10A.5F1	+	+++
				10A.5F2	+	+++
	10A.6	a	+++	10A.6F1	+++	+++
				10A.6F2	++	+++
				10A.6F3	+	+++
				10A.6F4	+++	+++
G10B (10 TgME49 oocysts)	**10B.1**	11	-	10B.1F1	-	-
				10B.1F2	-	-
	10B.2	a	+++	10B.2F1	++	+++
	10B.3	a	-	10B.3F1	-	-
				10B.3F2	-	-
	10B.4	a	+++	10B.4F1	+++	+++
	10B.5	a	+++	10B.5F1	+++	+++

[a] Day post-challenge when foetal death was detected by ultrasonography. The remaining ewes (*a*) delivered stillbirths/live lambs.

[b] Placentomes in ewes that aborted and cotyledons in ewes that gave birth;

γ Samples from foetal tissues exhibiting DNA degradation were excluded.

β Mummified foetuses were not evaluated.

dpi: days post-infection

NA: not available

Plus (+++, ++, +) and minus (-) signs represent PCR detection in >67%, 66-34%, <33% and 0% of samples analysed, respectively.

Figure S4 - Dot-plots showing number of lesions (A), individual focus area (B) and percentage of damaged area (C) in the brains from stillborn lambs and live lambs. Each dot represents individual, and medians are represented as horizontal lines.

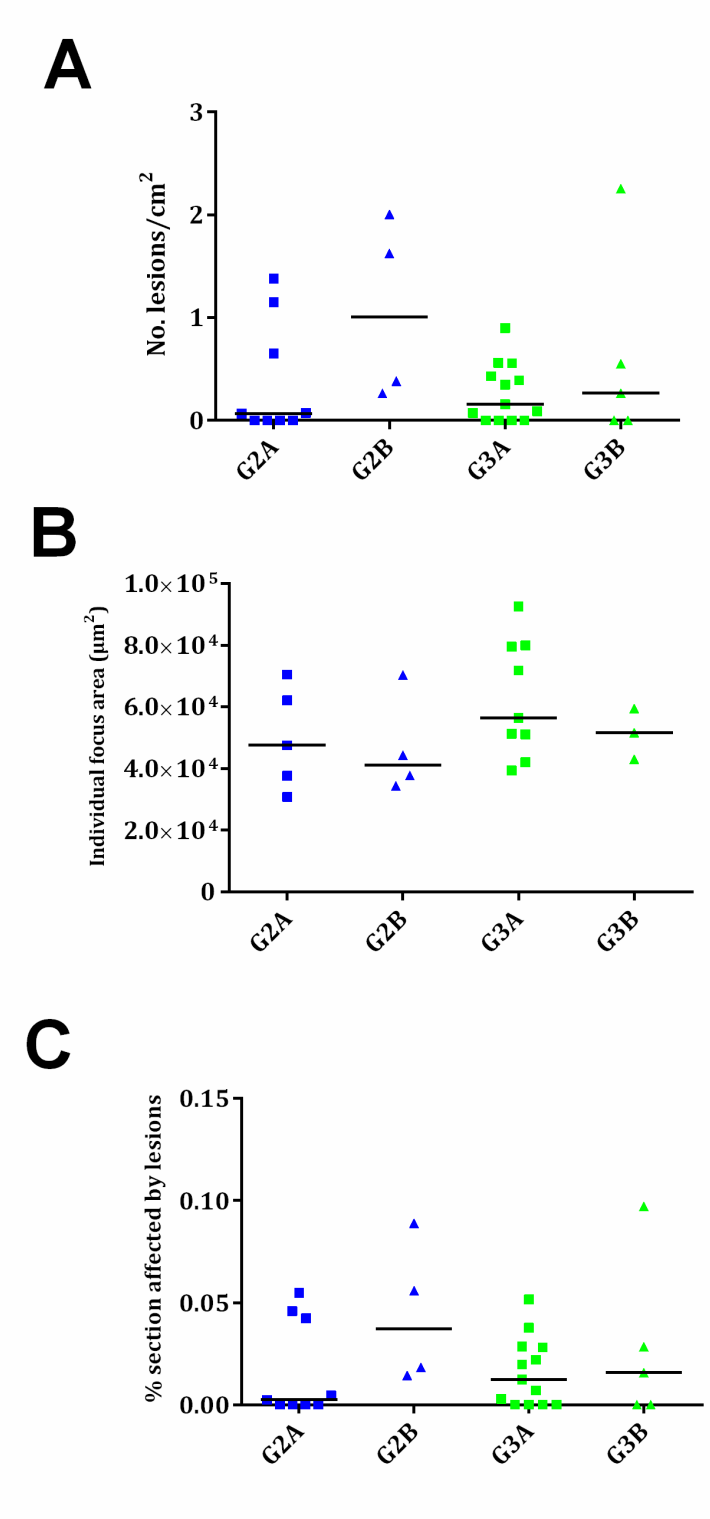


Table S3 - Individual serological titers in fetuses/lambs from infected ewes.

Group	Ewe ref.	Foetal death (dpi)[a]	Foetus/Lambs ref.	FL or Sera titre
G500A (500 TgShSp1 oocysts)	**500A.1**	9	500A.1F1	-
	500A.2	36	500A.2F1	1:64
	500A.3	8	500A.3F1	-
	500A.4	9	500A.4F1	-
			500A.4F2	-
			500A.4F3	-
	500A.5	8	500A.5F1	-
	500A.6	8	500A.6F1	-
			500A.6F2	-
			500A.6F3	-
			500A.6F4	-
			500A.6F5	-
G500B (500 TgME49 oocysts)	**500B.1**	8	500B.1F1	-
			500B.1F2	-
	500B.2	9	500B.2F1	-
			500B.2F2	-
			500B.2F3	-
	500B.3	8	500B.3F1	-
			500B.3F2	-
	500B.4	9	500B.4F1	-
	500B.5	9	500B.5F1	-
			500B.5F2	-
G50A (50 TgShSp1 oocysts)	**50A.1**	a	50A.1F1	-
			50A.1F2	1:8^
			50A.1F3	NA
	50A.2	10	50A.2F1	-
			50A.2F2	-
			50A.2F3	-
	50A.3	9	50A.3F1	-
			50A.3F2	-
	50A.4	a	50A.4F1	1:800
			50A.4F2	1:100
			50A.4F3	1:16^
	50A.5	a	50A.5F1	1:100
			50A.5F2	1:100
			50A.5F3	1:16^
	50A.6	10	50A.6F1	-
			50A.6F2	-
G50B (50 TgME49 oocysts)	**50B.1**	35	50B.1F1	1:128
	50B.2	a	50B.2F1	-^
	50B.3	11	50B.3F1	-
	50B.4	a	50B.4F1	1:400
			50B.4F2	1:200
	50B.5	a	50B.5F1	NA*

Table S3 – Continued.

Group	Ewe ref.	Foetal death (dpi)	Foetus/Lambs ref.	FL or Sera titre
G10A (10 TgShSp1 oocysts)	**10A.1**	49	10A.1F1	-
			10A.1F2	1:64
	10A.2	a	10A.2F1	1:100
			10A.2F2	1:100
	10A.3	a	10A.3F1	1:200
			10A.3F2	-
			10A.3F3	1:100
	10A.4	a	10A.4F1	-^
			10A.4F2	1:16^
	10A.5	a	10A.5F1	1:200
			10A.5F2	NA*
	10A.6	a	10A.6F1	1:200
			10A.6F2	1:100
			10A.6F3	1:800
			10A.6F4	-
G10B (10 TgME49 oocysts)	**10B.1**	11	10B.1F1	-
			10B.1F2	-
	10B.2	a	10B.2F1	1:50
	10B.3	a	10B.3F1	-
			10B.3F2	-^
	10B.4	a	10B.4F1	NA*
	10B.5	a	10B.5F1	1:50

[a] Day post-challenge when foetal death was detected by ultrasonography. The remaining ewes (*a*) delivered stillbirths/live lambs

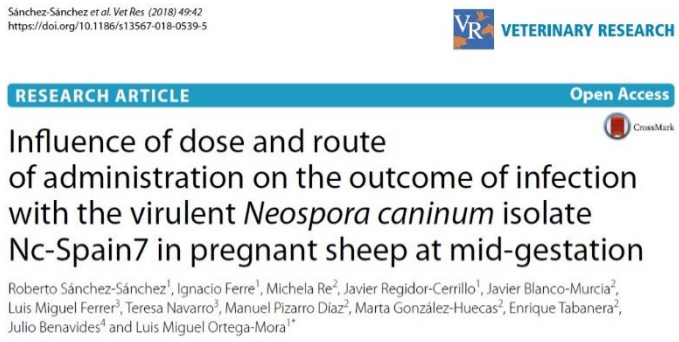
^ thoracic or abdominal fluids were analysed in stillborn lambs of which it was unable to obtain precolostral sera.

* suckling before serum sampling. dpi: days post-infection; FL: foetal liquid; NA: not available.

Objetivo 2

Estandarización de un modelo ovino gestante de infección con el aislado Nc-Spain7 de *N. caninum* mediante la evaluación de la influencia de la dosis y la via de administración

Las infecciones experimentales en ovejas gestantes se han centrado en el estudio del efecto que tiene el momento del desafío en el resultado de la infección por *N. caninum*, mientras que el impacto de la dosis y la vía de desafío no han sido estudiadas en profundidad. Asi pues, el resultado de la infección, la respuesta immune, la detección y carga del parásito y la gravedad de las lesiones en tejidos placentarios y cerebros fetales fueron investigadas en ovejas inoculadas por vía intravenosa en día 90 de gestación con 10^5, 10^4, 10^3 o 10^2 taquizoitos o por vía subcutánea con 10^4 taquizoitos del aislado virulento Nc-Spain7. Comparando las dosis de desafio, las ovejas desafiadas por vía intravenosa con 10^5 taquizoitos fueron las únicas que mostraron un 100% de aborto. Asimismo, los niveles de IFNγ en las ovejas desafíadas con 10^5 taquizoitos por vía intravenosa se incrementaron antes que en aquellas desafiadas por vía intravenosa con menos dosis, y los niveles de IgG en día 21 postinfección fueron mayores en las ovejas infectadas con 10^5 taquizoitos que en el resto de ovejas desafiadas por vía intravenosa con dosis menores. En relación a la transmisión vertical, las ovejas desafiadas con 10^5 taquizoitos por vía intravenosa mostraron una mayor carga parasitaria en el cerebro de los fetos que aquellas desafiadas con 10^4 y 10^3 taquizoitos por vía intravenosa. Comparando las vías de administración, no se observaron diferencias en la mortalidad fetal ni en la carga parasitaria de los cerebros de los fetos. Aunque las ovejas desafiadas con 10^4 taquizoitos por vía intravenosa mostraron mayores niveles de IFNγ que aquellas desafiadas con 10^4 taquizoitos por vía subcutánea, no se vieron diferencias en las respuestas immunes humorales. Dado que el resultado del desafio por vía intravenosa con 10^5 taquizoitos es similar al observado en un estudio previo tras la infección por vía intravenosa con 10^6 taquizoitos (100% de aborto y transmisión vertical), se concluye que quizás sea razonable el uso de 10^5 taquizoitos por vía intravenosa en futuros experimentos que evalúen candidatos farmacológicos y vacunales.



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Published in *Veterinary Research* (8th May 2018) 49:42. DOI: 10.1186/s13567-018-0539-5. Presented as poster communication in the 4th International Meeting on Apicomplexa in Farm Animals (*Apicowplexa*), (11th-14th October 2017, Madrid, Spain)

Abstract

Experimental infections in pregnant sheep have been focused on studying the effect of the time of challenge on the outcome of *N. caninum* infection, whereas the impact of the dose and route of challenge has not been studied in depth. Therefore, clinical outcome, immune responses, parasite detection and burden, and lesion severity in placental tissues and foetal brains were investigated in 90-day-pregnant sheep inoculated intravenously with 10^5 (G1), 10^4 (G2), 10^3 (G3), or 10^2 (G4) tachyzoites or subcutaneously with 10^4 (G5) tachyzoites of the virulent Nc-Spain7 isolate and an uninfected group (G6). Comparing challenge doses, G1 was the only group that showed 100% abortion. Likewise, IFNγ levels in G1 increased earlier than those in other intravenously infected groups, and IgG levels on day 21 post-infection (pi) were higher in G1 than those in other intravenously infected groups. Concerning vertical transmission, G1 showed a higher parasite burden in the foetal brain than did G2 and G3. Comparing routes of administration, no differences in foetal survival rate or parasite load in the foetal brain were found. Although G2 showed higher IFNγ levels than G5 on day 10 pi, no differences were found in humoral immune responses. Because the outcome after intravenous infection with 10^5 tachyzoites is similar to that observed after intravenous infection with 10^6 tachyzoites used in a previous work (100% abortion and vertical transmission), we conclude that it may be reasonable to use 10^5 tachyzoites administered by the intravenous route in further experiments when assessing drugs or vaccine candidates.

Keywords: *Neospora caninum*, pregnant sheep model, Nc-Spain7, dose titration, route of administration, abortion, vertical transmission

1. Introduction

Neospora caninum is an obligate intracellular apicomplexan parasite considered one of the leading infectious causes of abortion in cattle worldwide (Dubey *et al.*, 2007; Reichel *et al.*, 2013; Dubey *et al.*, 2017). Recent studies suggest that *N. caninum* could also be a relevant abortifacient in some small ruminant management systems (Moreno *et al.*, 2012) or even the main cause of reproductive losses in some flocks (West *et al.*, 2006; González-Warleta *et al.*, 2014). The pathogenesis of ovine neosporosis is poorly understood and, in contrast to the clinical outcome in cattle, infection during mid-pregnancy in sheep resulted in severe clinical outcome, as most of the animals aborted or, less frequently, produced weak lambs (McAllister *et al.*, 1996b; Buxton *et al.*, 1998; Arranz-Solis *et al.*, 2015b).

In pregnant sheep, infective doses of 10^7-10^8 tachyzoites resulted in a high percentage of abortions (Dubey and Lindsay, 1990; Buxton *et al.*, 2001; Innes *et al.*, 2001a; Weston *et al.*, 2009). In a study comparing different infective doses, a strong relationship between the challenge dose of Nc-NZ1, Nc-NZ2 and Nc-NZ3 *N. caninum* tachyzoites and the clinical outcome was found in pregnant sheep at mid-gestation (Weston *et al.*, 2009). To date, there are no studies comparing the outcome of *N. caninum* experimental infection using different routes of inoculation in pregnant sheep, although, in cattle, it was crucial because intravenous inoculation was associated with a more severe clinical presentation than subcutaneous inoculation (Macaldowie *et al.*, 2004). Likewise, there are clear differences concerning the outcome of the infection among parasite isolates (Benavides *et al.*, 2014). The Nc-Spain7 isolate (Regidor-Cerrillo *et al.*, 2008) is a very well-characterized virulent isolate tested so far in three experimental ruminant models, sheep (Arranz-Solis *et al.*, 2015b), goats (Porto *et al.*, 2016) and cattle (Caspe *et al.*, 2012; Regidor-Cerrillo *et al.*, 2014; Almería *et al.*, 2016).

Recently, the Nc-Spain7 isolate has been evaluated at different times during gestation in pregnant sheep, suggesting that the time of infection plays a key role in the pathogenesis of the disease (Arranz-Solis *et al.*, 2015b).

Therefore, the aim of this study was to investigate the effect of challenge dose and route of administration in the outcome of experimental infection in ewes at mid-term of gestation using the Nc-Spain7 isolate. The effect of parasite dose and route of administration on the clinical course of disease, cellular and humoral immune responses, lesion development and parasite detection and burden in placental and foetal tissues were evaluated. This experiment allowed the refinement and standardization of an exogenous transplacental transmission model for ovine neosporosis.

2. Material and methods

2.1. Ethics statement

All protocols involving animals were approved by the Animal Welfare Committee of the Community of Madrid, Spain following procedures described in Spanish and EU legislations (PROEX 93/14, Law 32/2007, R.D. 53/2013, and Council Directive 2010/63/EU). All animals used in this study were handled in strict accordance with good clinical practices, and all efforts were made to minimize suffering.

2.2. Animals and experimental design

Forty Rasa Aragonesa breed female lambs aged 3 months were selected from a commercial flock after checking their seronegativity for *T. gondii*, *N. caninum*, Border disease virus (BDV), Schmallenberg virus (SBV), *Coxiella burnetii* and *Chlamydia abortus* by ELISA. Animals were maintained in isolation at Zaragoza University (Spain) facilities and at 12 months old were oestrus synchronized by insertion of intravaginal progestogen-impregnated sponges (Chronogest® 20 mg fluorogestone acetate, MSD Animal Health, Salamanca, Spain) for 14 days. At the time of removal, 480 UI of

pregnant mare serum gonadotrophin (PMSG) (Foligon® 6000 UI, MSD Animal Health, Salamanca, Spain) was administered to each ewe through intramuscular injection as previously described Abecia *et al.* (2011). After 48 hours, ewes were mated with breed Rasa Aragonesa tups for 2 days, after which, the rams were removed from the ewes. Pregnancy and foetal viability were confirmed by ultrasound scanning (US) on day 40 after mating, and twenty-seven pregnant sheep were selected for the experiment. Pregnant ewes (n=27) were randomly distributed into six experimental groups at Clinical Veterinary Hospital facilities of Complutense University of Madrid (Spain). Twenty-four ewes were inoculated intravenously into the jugular vein at 90 days of gestation (dg) with 10^5 (group 1, G1; n=6), 10^4 (group 2, G2; n=5), 10^3 (group 3, G3; n=5), 10^2 (group 4, G4; n=4) tachyzoites; or subcutaneously over the left prefemoral lymph node with 10^4 tachyzoites (group 5, G5; n=4) of the Nc-Spain7 bovine isolate (Regidor-Cerrillo *et al.*, 2008). The three remaining pregnant ewes were allocated to group 6 (G6; n=3), acted as uninfected controls and received an intravenous inoculum of phosphate-buffered saline (PBS) at 90 dg (Table 1).

2.3. Parasite culture and dose preparation

Tachyzoites of the Nc-Spain7 isolate were routinely maintained in cultured MARC-145 cells as described previously (Regidor-Cerrillo *et al.*, 2010). For the challenge, tachyzoites (passage 19) were recovered from culture flasks when they were still largely intracellular (>80% of undisrupted parasitophorous vacuoles), and infected cells were repeatedly passed through a 25-gauge needle at 4ºC. The number of viable tachyzoites was determined by Trypan blue exclusion (typically 95–99%) followed by counting the viable tachyzoites in a Neubauer chamber. Subsequently, the concentration of viable tachyzoites was adjusted to the required dose (10^5, 10^4, 10^3 and 10^2) by dilution in PBS in a final volume of 1 mL. Tachyzoites were administered to pregnant ewes within 30 min of harvesting from cell culture.

2.4. Clinical monitoring and collection of samples

Pregnant ewes were observed daily throughout the entire experimental period. Rectal temperatures were recorded daily from day 0 until 14 days pi and then weekly. Animals were considered febrile when the rectal temperature was over 40ºC (Diffay *et al.*, 2002).

In G5, which was subcutaneously inoculated with Nc-Spain7 tachyzoites, changes in the left prefemoral lymph node compared to the right prefemoral lymph node by palpation were recorded daily until its resolution. The left prefemoral lymph node was regarded as enlarged if its size exceeded that of the right prefemoral lymph node by at least 50%.

Blood samples to evaluate immune responses were collected before infection, on days 3, 5, 7 and 10 pi and then weekly by jugular venipuncture into 5 mL Vacutainer tubes (Becton Dickinson and Company, Plymouth, UK) with lithium heparin as anticoagulant and without anticoagulant. Tubes without anticoagulant were allowed to clot and centrifuged to obtain serum, and samples were stored at −80°C until analysis.

Foetal viability was assessed by transabdominal ultrasonography (US) to monitor foetal heartbeat and movements once weekly for the first two weeks post-infection (pi) and then twice weekly until detection of foetal death. When foetal death was detected in any of the foetuses or 24 hours after parturition, dams and lambs were first sedated with

Table 1 - Experimental design.

Group	Number of pregnant ewes	Number of foetuses	Ratio foetuses/dam	Inoculum	Route of inoculation
G1	6	12	2	Nc-Spain7 10^5 tachyzoites	IV
G2	5	11	2.20	Nc-Spain7 10^4 tachyzoites	IV
G3	5	13	2.60	Nc-Spain7 10^3 tachyzoites	IV
G4	4	7	1.75	Nc-Spain7 10^2 tachyzoites	IV
G5	4	8	2	Nc-Spain7 10^4 tachyzoites	SC
G6	3	5	1.66	PBS	IV

IV: intravenous route
SC: subcutaneous route

xylazine (Rompun, Bayer, Mannhein, Germany) and then immediately euthanized by an IV overdose of embutramide and mebezonium iodide (T61, Intervet, Salamanca, Spain).

At necropsy, six randomly selected placentomes were recovered from each placenta of aborted dams, were transversally cut into slices of 2–3 mm thickness and were stored in 10% formalin for histopathological examinations. The rest of the placentomes were stored at −80 °C for further parasite DNA detection by PCR. In dams that gave birth, six randomly selected cotyledons were recovered and stored at -80 ºC for further parasite DNA detection by PCR. From foetuses, the foetal brain was stored at −80°C for DNA extraction and fixation in 10% formalin. Foetal thoracic and abdominal fluids or precolostral serum were also collected from aborted foetuses or newborn lambs, respectively, and maintained at −80°C for serology.

Lambs were weighed and sampled for blood at birth and were euthanized 24 hours after birth. To avoid any accidental suckling from lambs born overnight, udders were covered with a piece of cloth one week before the expected date of delivery as a preventive measure.

2.5. Peripheral blood stimulation assay and interferon-gamma (IFNγ) production analysis

Peripheral blood stimulation assay was carried out and interferon-gamma (IFNγ) production was evaluated as previously described Sánchez-Sánchez *et al.* (2018). Briefly, heparinised blood was cultured in 24-well flat-bottom plates in the presence of either soluble *N. caninum* antigens or concanavalin A (ConA, Sigma-Aldrich, Madrid, Spain), both at final concentrations of 5 μg/mL. Plates were incubated in a 5% CO_2/37°C/100% humidity atmosphere for 24 h. They were then centrifuged at 1000 x *g* for 10 min at 4°C, and culture supernatants were assayed for IFN-γ detection using a commercial bovine enzyme

immunoassay kit with a capture monoclonal antibody (MT17.1) showing cross-reactivity with ovine IFNγ (Mabtech AB, Sweden) as previously described Arranz-Solis *et al.* (2015b).

2.6. Serological analyses: ELISA and IFAT

Neospora caninum-specific IgG antibody levels were measured using an in-house indirect enzyme-linked immunosorbent assay (ELISA) as previously described Sánchez-Sánchez *et al.* (2018). Briefly, 96-well microtiter plates (Thermo Fisher Scientific, Waltham, USA) were coated with 100 µL soluble *N. caninum* antigen (1 µg/mL in 100 mM carbonate buffer pH 9.6) overnight at 4°C (Alvarez-Garcia *et al.*, 2003). Plates were blocked and serum samples were diluted 1:100 using 3% bovine serum albumin diluted in PBS containing 0.05% Tween 20 (PBS-T). Subsequently, horseradish peroxidase-conjugated protein G (Sigma-Aldrich, Madrid, Spain) diluted 1:2000 in PBS-T was added and after that, ABTS (Roche, Basilea, Switzerland) was used as substrate. The reaction was stopped by 0.3 M oxalic acid and the optical density (OD) was read at 405 nm (OD405). For each plate, values of the OD were converted into a relative index percent (RIPC) using the following formula: RIPC = (OD405 sample – OD405 negative control)/(OD405 positive control–OD405 negative control) × 100. A RIPC value ≥10 indicates a positive result.

An indirect fluorescent antibody test (IFAT) was used to detect specific IgG anti-*Neospora* antibodies in foetal fluids and precolostral sera, according to the technique described by Alvarez-Garcia *et al.* (2003) and used in previous studies (Sánchez-Sánchez *et al.*, 2018).

2.7. Histopathology and lesion scoring

After fixation in formalin for five days, formalin-fixed samples were cut, embedded in paraffin wax, and processed by standard procedures for haematoxylin and eosin (HE) staining. Conventional histological evaluation was carried out on all the sections. The analysis was based on the observation of lesions according to previous descriptions (Arranz-Solis *et al.*, 2015b), and lesions were classified as none detected/unrelated (-), mild lesions (+) (in the placentome, 0-33.3% of the placentome showed lesions; in the foetal brain, presence of local encephalitis), moderate lesions (++) (in the placentome, 33.3-66.6% of the placentome showed lesions; in the foetal brain, presence of diffuse encephalitis) and severe lesions of ovine neosporosis (+++) (in the placentome, 66.6-99.9% of the placentome showed lesions; in the foetal brain, presence of diffuse encephalitis and necrosis).

2.8. DNA extraction and PCR for parasite detection and quantification in tissues

Genomic DNA was extracted from 50–100 mg of maternal and foetal tissue samples using the commercial Maxwell® 16 Mouse Tail DNA Purification Kit, developed for the automated Maxwell® 16 System (Promega, Wisconsin, USA), following the manufacturer's recommendations. The concentration of DNA for all samples was determined by spectrophotometry and adjusted to 50–100 ng/µL.

PCR was carried out on six placentome samples from aborted dams or cotyledons in dams that gave birth and three foetal brain samples. Parasite DNA detection was carried out by nested PCR adapted to a single tube as previously described Buxton (1998) and Regidor-Cerrillo *et al.* (2014). Each reaction was performed in a final volume of 25 µL with 5 µL of sample DNA. Samples from the uninfected group (G6) were included in each round of DNA extraction and PCR as negative controls. Positive PCR controls with *N. caninum* genomic DNA equivalent to 10, 1 and 0.1 tachyzoites in 100 ng of sheep DNA were also included in each batch of amplifications. Ten microlitre aliquots of the PCR products were visualized under UV light in a 1.5%

agarose/ethidium bromide gel to detect the *N. caninum*-specific 247 bp amplification product.

Placenta and foetal brain samples that tested positive by nested-PCR were adjusted to 20 ng DNA/μL, and the parasite load was quantified using real-time PCR. Primer pairs from the *N. caninum* Nc-5 sequence (Collantes-Fernández et al., 2002) were used for parasite quantification, and primers from the β-actin gene (Gutierrez *et al.*, 2012) were used for the quantification of host DNA. Amplification reactions were performed as described by Sánchez-Sánchez *et al.* (2018).

2.9. Statistical analysis

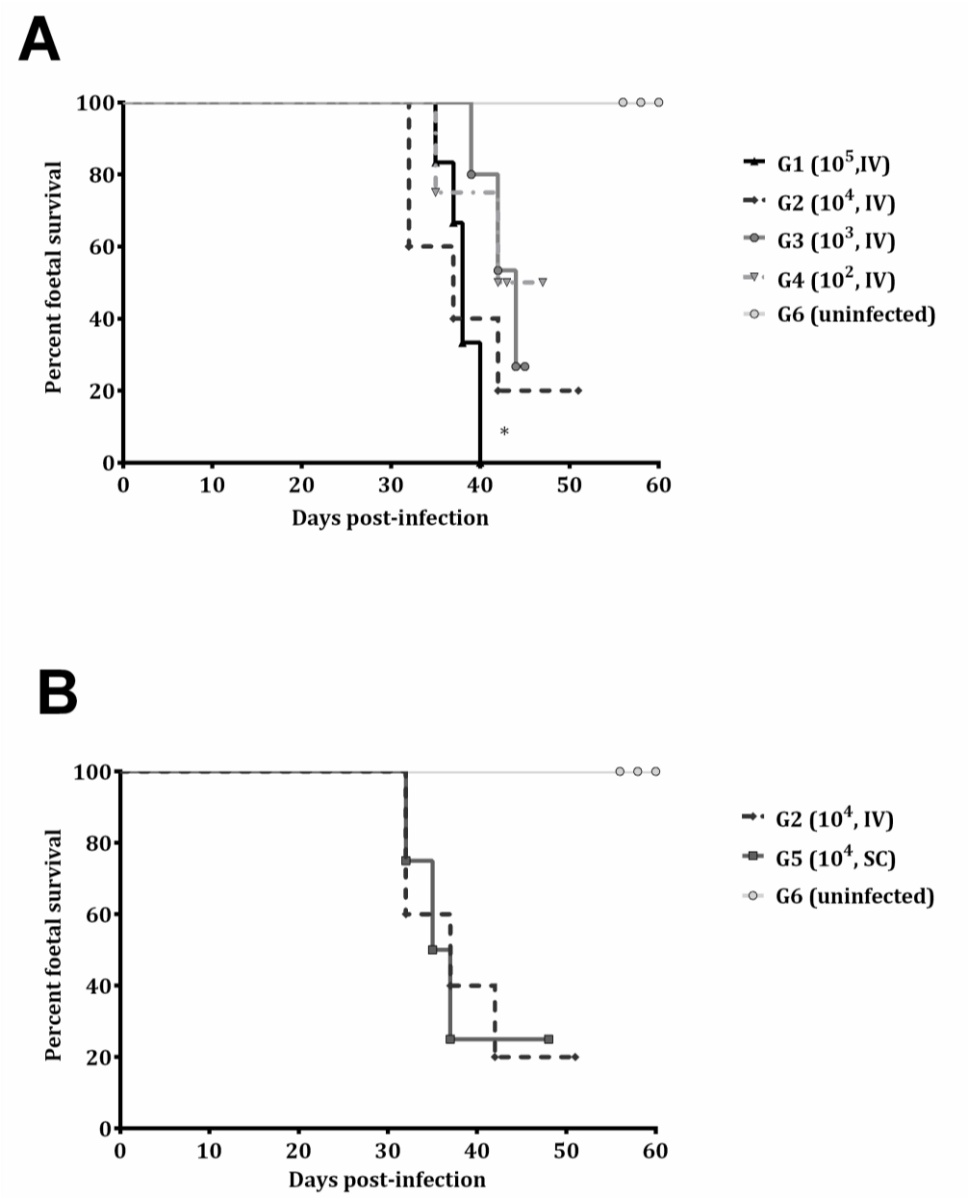
The occurrence of foetal death was analysed by the Kaplan–Meier survival method. Foetal survival curves were then compared by the Log-rank (Mantel-Cox) test, and the median foetal survival time, i.e., the day at which 50% of the foetuses aborted, was calculated. Weight of the lambs and antibody responses in foetuses and lambs were compared using the non-parametric Kruskal–Wallis test followed by Dunn's test for comparisons between groups and the Mann–Whitney test for pairwise comparisons. Rectal temperatures were analysed using a two-way ANOVA of repeated measures test until 14 days pi and a one-way ANOVA test afterwards. Humoral and cellular immune responses for each experimental group were analysed using a two-way ANOVA of repeated measures test until 28 days pi and a one-way ANOVA test afterwards. However, cellular immune responses in G1 were analysed using one-way ANOVA test. Differences in PCR detection of parasite DNA were evaluated using the $\chi 2$ or Fisher Exact F-test. Differences in parasite burdens and lesion severity were analysed using the non-parametric Kruskal–Wallis test followed by Dunn's test for comparisons between groups and the Mann–Whitney test for pairwise comparisons. Statistical significance for all analyses was established at $P < 0.05$. Differences that showed P values ≥ 0.05 and ≤ 0.18 were considered to be trending towards statistical signification. All statistical analyses were carried out using GraphPad Prism 6.01 software (San Diego, CA, USA).

3. Results

3.1. Clinical observations

Foetal death was detected from 32-44 days pi by US in 6 out of 6 pregnant ewes in G1 (median abortion day 38), 4 out of 5 pregnant ewes in G2 (median abortion day 34), 3 out of 5 pregnant ewes in G3 (median abortion day 42), 2 out of 4 pregnant ewes in G4 (median abortion day 38) and 3 out of 4 pregnant ewes in G5 (median abortion day 35) (Table 2). The median survival times were 38, 37, 44, 51 and 36 days for G1, G2, G3, G4 and G5, respectively (Figure 1). All aborted dams in G3 and G4, one aborted dam in G1 and one aborted dam in G5 had twin pregnancies, and they showed, before being euthanized, foetal death in one of the foetuses and foetal heartbeat and movements by US in the other. One mummified foetus in G1 and three mummified foetuses in G2 were found at necropsy (Table 2). All dams from the uninfected group (G6) gave birth, and a significant difference was found in the foetal survival rate compared to that in G1 ($P < 0.05$). Regarding challenge doses, compared to G1, a higher foetal survival rate was found in G3 ($P < 0.05$), and in G4, the statistical difference ($P = 0.06$) showed a trend towards significance (Figure 1A). Regarding routes of administration, no significant differences in the foetal survival rate between G2 and G5 were found (Figure 1B). Non-aborted dams in G3, G4 and G5 gave birth

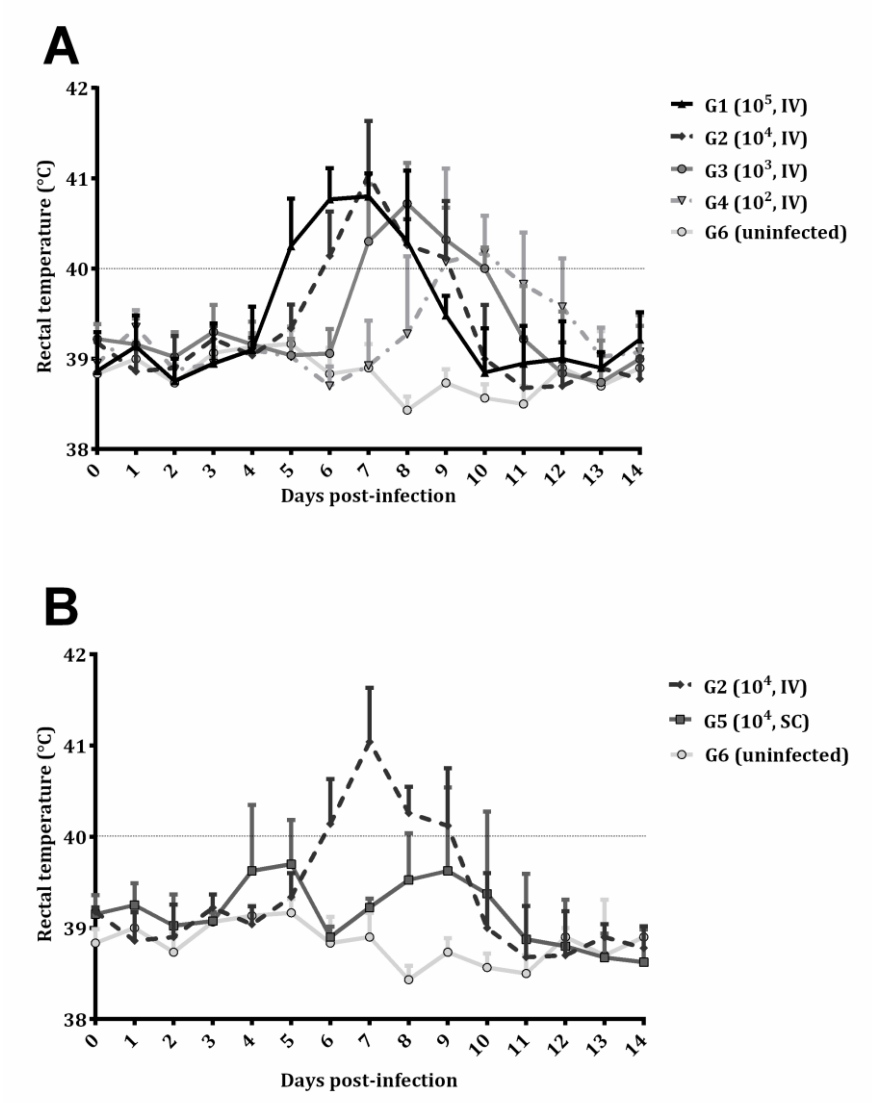
Figure 1 - Kaplan–Meier survival curves for foetuses from intravenously challenged dams and the uninfected group (A) and intravenously and subcutaneously challenged pregnant ewes with 10^4 Nc-Spain7 tachyzoites and the uninfected group (B). Each point represents the percentage of surviving animals on that day, and downward steps correspond to observed deaths. Foetal survival curves were compared by the log-rank (Mantel-Cox) test. For significant differences between foetal survival curves of infected groups, (*) indicates $P < 0.05$.



prematurely between days 132 and 141 of pregnancy. Stillborn lambs and lambs exhibiting weakness, recumbency and unresponsiveness to external stimuli and dying within 24 after birth were found in 7 of 7 lambs in G3, 2 of 3 lambs in G4 and G5. In contrast, non-aborting dams in G2 and dams in G6 gave birth to healthy lambs between days 144 and 150 of pregnancy. A significant decrease in the weight of the lambs from G3 (1511.5 ± 85.5 g) ($P < 0.01$), G4 (2429 ± 447.4 g) ($P < 0.05$) and G5 (2213.3 ± 528.7 g) ($P < 0.05$) was found compared to that in G6 (4037.4 ± 354.7 g), whereas no statistical analysis was performed in G2 since only one lamb was born (2919 g).

Concerning rectal temperatures, all intravenously challenged animals showed fever (rectal temperature above 40ºC) at some time point until day 14 pi. Compared to the uninfected group (G6), a significant increase in rectal temperature was found from day 5 pi ($P < 0.01$) to day 8 pi ($P < 0.0001$) in G1, from day 6 pi ($P < 0.001$) to day 9 pi ($P < 0.001$) in G2, from day 7 pi ($P < 0.001$) to day 10 pi ($P < 0.0001$) in G3 and from day 9 pi ($P < 0.001$) to day 11 pi ($P < 0.001$) in G4, with maximum mean rectal temperature on day 7 pi in G1 and G2, on day 8 pi in G3 and on day 10 pi in G4 (Figure 2A). Likewise, in the subcutaneously challenged group (G5), a significant increase in rectal temperature on day 8 pi ($P < 0.05$) was found when compared to that in the uninfected group (G6); moreover, significant increases in rectal temperatures on days 4 ($P < 0.05$), 5 ($P < 0.05$) and 9 pi ($P < 0.05$) were found compared to that on day 14 pi in G5.

Figure 2 - Rectal temperatures of intravenously challenged pregnant ewes and the uninfected group (A) and intravenously and subcutaneously challenged pregnant ewes with 10^4 Nc-Spain7 tachyzoites and the uninfected group (B). Each point represents the mean + SD at different times for each group. Rectal temperatures represented in the figure were analysed using two-way ANOVA of repeated measures.



In contrast to the intravenously infected groups, no mean rectal temperature above 40ºC was found in the subcutaneously challenged group (G5) at any time, and only a few animals showed fever (one ewe on days 4, 5, 8 and 10 pi and two ewes on day 9 pi) (Figure 2B). Comparing rectal temperatures between aborting ewes and ewes that gave birth, significant differences were found only in G4 since aborting ewes showed higher rectal temperatures on days 8 ($P < 0.05$) and 9 pi ($P < 0.001$) than ewes that gave birth. Likewise, in G4, aborting ewes showed fever on days 9 and 10 pi, whereas ewes that gave birth showed fever on day 11 pi. The mean rectal temperature in the uninfected group (G6) remained below 39.5ºC throughout the monitoring period. From day 14 pi until the end of the experiment, no changes were found in rectal temperatures in the infected groups.

In G5, enlargement of the left prefemoral lymph node was observed in all pregnant sheep between days 2 and 14 pi.

3.2. Cellular and humoral immune responses

IFNγ levels in supernatants recovered 24 hours after *N. caninum* antigen stimulation are shown in Figure 3. IFNγ values increased on day 7 pi in G1 ($P < 0.05$) and on day 10 pi in G2 ($P < 0.0001$), G3 ($P < 0.0001$) and G5 ($P < 0.05$), whereas G4 did not show a significant increase in IFNγ at any time pi compared to the uninfected group (G6). Regarding challenge doses, on day 10 pi, G2 showed higher IFNγ than G4 ($P < 0.0001$) (Figure 3A). Regarding routes of administration, on day 10 pi, G2 showed higher IFNγ than G5 ($P < 0.001$) (Figure 3B). The ewe that gave birth in G2 showed higher IFNγ on day 10 pi than those that aborted ($P < 0.01$), but ewes that gave birth in G3 showed lower IFNγ than those that aborted ($P < 0.001$). In G4, aborting ewes showed higher IFNγ on day 10 pi than those that gave birth ($P < 0.05$); however, ewes that gave birth showed higher IFNγ on day 14 pi ($P < 0.05$) than those that aborted. No significant differences between ewes that gave birth and aborting ewes were found in G5. No significant differences between groups were found in IFNγ levels from days 14 to 28 pi. Likewise, all infected groups maintained low IFNγ levels from day 28 pi onwards (data not shown). None of the uninfected animals (G6) showed IFNγ levels above basal levels recorded prior to inoculation throughout the experimental study.

The *N. caninum*-specific IgG antibody response in dams is shown in Figure 4. When compared to those in the uninfected group (G6), IgG levels increased significantly from day 21 pi in G1 ($P < 0.0001$), G2 ($P < 0.01$) and G5 ($P < 0.05$) and from day 28 pi in G3 ($P < 0.001$) and G4 ($P < 0.05$). Moreover, when challenge doses were compared, G1 showed higher IgG values on day 21 pi than all other intravenously infected groups ($P < 0.0001$) and on day 28 pi than G3 ($P < 0.0001$) and G4 ($P < 0.0001$). Likewise, G2 showed higher IgG values than G4 on days 21 and 28 pi ($P < 0.05$) (Figure 4A). From day 28 pi until foetal death/birth occurred, G1 showed higher IgG values than G2 ($P < 0.01$), G3 ($P < 0.01$) and G4 ($P < 0.01$) (data not shown). When routes of administration were compared, no significant differences in IgG serum levels were found between G2 and G5 at any time (Figure 4B). Likewise, no significant differences were found between aborting ewes and ewes that gave birth in any of the infected groups. Seroconversion in all animals from infected groups was observed at the time of abortion/birth. All uninfected animals (G6) showed basal IgG levels throughout the experimental study.

The *Neospora*-specific IgG response in foetal fluids from aborted foetuses and stillborn lambs or precolostral sera collected from lambs is summarized in Table 2. Seropositive titres were detected in all aborted foetuses from infected groups, ranging from 1:32 to 1:1024. IFAT titre medians in aborted foetuses were 1:128 for G2, 1:256 for G1, G3 and G5, and 1:512 for G4 without significant differences

Figure 3 - IFNγ response in the supernatants of intravenously challenged pregnant ewes and the uninfected group (A) and pregnant ewes intravenously and subcutaneously challenged with 10^4 Nc-Spain7 tachyzoites and the uninfected group (B). Each point represents the mean + SD at the different sampling times for each group. IFNγ levels from G1 on day 10 pi are not available due to problems in antigen stimulation assays of the peripheral blood. Data beyond day 28 pi are not included because several animals did not maintain pregnancy and were therefore sacrificed. Concentrations of IFNγ are expressed in pg/mL. The cellular immune response represented in the figure was analysed using two-way ANOVA of repeated measures. For significant differences between infected groups, (***) indicates $P < 0.001$, and (****) indicates $P < 0.0001$.

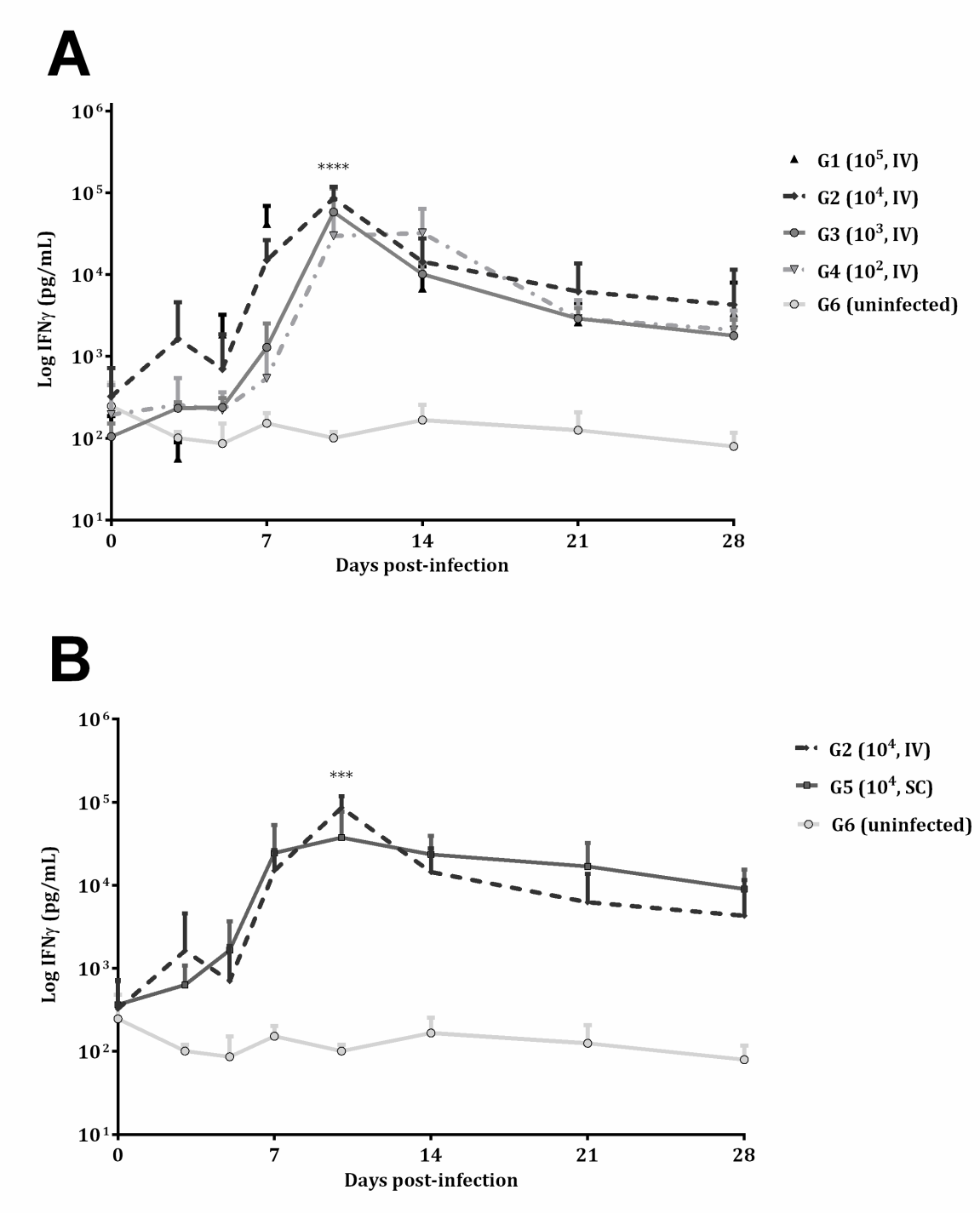


Figure 4 - IgG response in the serum of intravenously challenged pregnant ewes and the uninfected group (A) and pregnant ewes intravenously and subcutaneously challenged with 10^4 Nc-Spain7 tachyzoites and the uninfected group (B). Each point represents the mean + SD at the different sampling times for each group. Data beyond day 28 pi are not included because several animals did not maintain pregnancy and were therefore sacrificed. Serum levels of total IgG antibodies against *N. caninum* are expressed as a relative index percent (RIPC), according to the formula: RIPC = (OD405 sample– OD405 negative control)/(OD405 positive control–OD405 negative control) × 100. The humoral immune response shown in the figure was analysed using two-way ANOVA of repeated measures. For significant differences between infected groups, (*) indicates $P < 0.05$ and (****) indicates $P < 0.0001$.

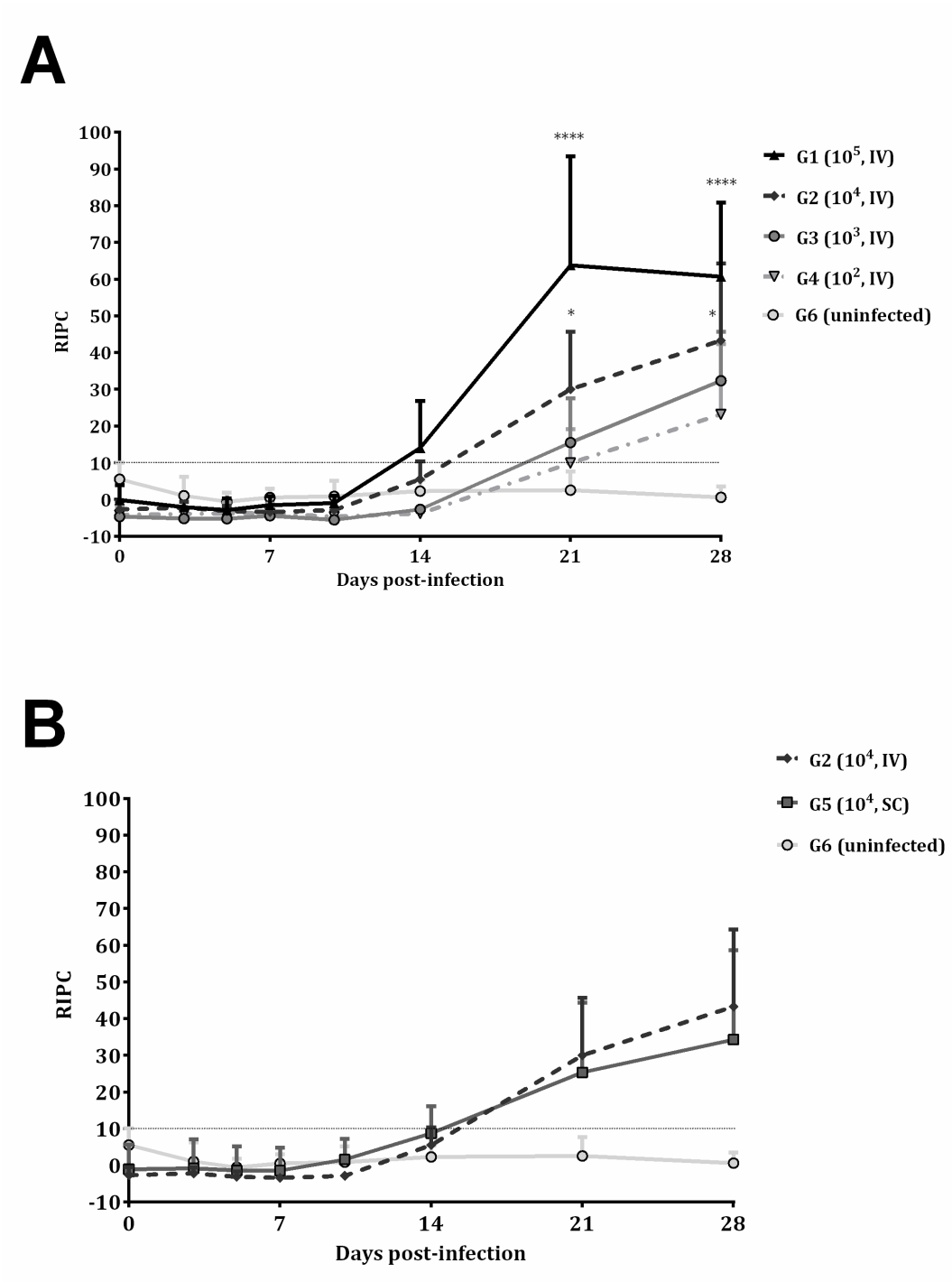


Table 2 - Detection of parasite DNA, parasite load and histopathological changes in foetal brains and placental tissues from sheep and serological analysis of the foetuses after challenge infection with the Nc-Spain7 isolate at mid-gestation.

Group	Animal id.	Foetal death (days p.i.)[a]	Placentomes/cotyledons[b]			Foetal brain															
						Foetus/lamb 1				Foetus/lamb 2				Foetus/lamb 3				Foetus/lamb 4			
			HP[c]	DNA[e]	qPCR[f]	IFAT[d]	HP[c]	DNA[e]	qPCR[f]	IFAT[d]	HP[c]	DNA[e]	qPCR[f]	IFAT[d]	HP[c]	DNA[e]	qPCR[f]	IFAT[d]	HP[c]	DNA[e]	qPCR[f]
Group 1 (10^5 tachyzoites, IV)	1.1	38	+++	6/6	2030.18 ± 2021.53	1:32	+++	3/3	47.53 ± 22.41	1:256	*	0/3	0.01								
	1.2	40	+++	6/6	1990.74 ± 543.90	1:256	++	2/3	3.40 ± 3.01												
	1.3	40	++	6/6	3355.17 ± 2407.07	1:1024	+++	3/3	8.47 ± 2.67												
	1.4	37	++	6/6	2028.50 ± 2434.11	1:128	++	2/3	14.42 ± 13.01	1:256	+++	3/3	584.97 ± 403.74	1:32	*	3/3	97.72 ± 109.14	β	β	β	β
	1.5	35	+++	6/6	3296.38 ± 1231.41	1:64	+++	3/3	141.14 ± 69.34	1:32	*	3/3	56.18 ± 27.50								
	1.6	38	+++	6/6	7974.29 ± 4660.05	1:1024	++	2/3	13.26 ± 15.57	1:1024	++	3/3	16.86 ± 10.67								
Group 2 (10^4 tachyzoites, IV)	2.1	32	+++	6/6	7420.68 ± 6570.82	1:256	++	3/3	13.38 ± 6.04												
	2.2		NA	4/4[γ]	3992.48 ± 1562.24[γ]	1:800	+	2/3	5.16 ± 5.59	β	β	β	β	β	β	β	β				
	2.3	32	+++	6/6	4548.31 ± 4733.95	1:128	+	3/3	19.61 ± 12.04												
	2.4	37	+	6/6	1930.36 ± 2034.10	1:256	++	2/3	5.54 ± 6.06	β	β	β	β								
	2.5	42	+++	6/6	6829.41 ± 3917.31	1:64	+	3/3	2.70 ± 0.86	1:64	++	3/3	20.29 ± 8.74	NA	++	3/3	11.97 ± 10.24	1:128	++	3/3	3.31 ± 2.49
Group 3 (10^3 tachyzoites, IV)	3.1	39	++	6/6	12260.49 ± 9266.82	1:32	++	3/3	9. 48 ± 5.47	1:256	++	3/3	23.56 ± 13.52								
	3.2	42	++	6/6	7370.42 ± 9178.25	1:256	++	3/3	2.27 ± 0.73	1:512	++	0/3	0.01								
	3.3	44	++	6/6	2307.22 ± 1754.57	1:256	++	3/3	39.60 ± 43.86	1:128	++	0/3	0.01								
	3.4		NA	5/6	62.07 ± 95.88	1:400	++	2/3	3.07 ± 2.72	1:400	++	2/3	3.33 ± 3.52	1:200	+	0/3	0.01	1:256	+	1/3	0.67 ± 1.15
	3.5		NA	6/6	823.41 ± 714.63	1:1024	++[α]	3/3	7.74 ± 3.20	1:256	+	3/3	6.54 ± 0.30	1:64	++	3/3	15.91 ± 7.92				

Table 2 - Continued.

Group	Animal id.	Foetal death (days p.i.)[a]	Placentomes/cotyledons[b]			Foetal brain															
						Foetus/lamb 1				Foetus/lamb 2				Foetus/lamb 3				Foetus/lamb 4			
			HP[c]	DNA[e]	qPCR[f]	IFAT[d]	HP[c]	DNA[e]	qPCR[f]	IFAT[d]	HP[c]	DNA[e]	qPCR[f]	IFAT[d]	HP[c]	DNA[e]	qPCR[f]	IFAT[d]	HP[c]	DNA[e]	qPCR[f]
Group 4 (10^2 tachyzoites, IV)	4.1		NA	6/6	1334.88 ± 561.06[γ]	1:400	+	3/3	12.44 ± 7.18	1:64	*	3/3	37.24 ± 17.02								
	4.2		NA	6/6	6160.29 ± 4646.09[γ]	1:400	+	1/3	1.22 ± 2.11												
	4.3	35	++	6/6	2101.24 ± 1290.86[γ]	1:512	++	3/3	23.03 ± 3.12	1:1024	++	3/3	14.35 ± 12.77								
	4.4	42	++	6/6	3226.22 ± 2350.13[γ]	1:512	++	3/3	45.58 ± 22.09	1:128	++	3/3	20.07 ± 13.80								
Group 5 (10^4 tachyzoites, SC)	5.1		NA	6/6	65.40 ± 27.49[γ]	1:800	+	3/3	3.87 ± 1.85	1:200	+	1/3	0.65 ± 1.12	1:800	+	3/3	7.22 ± 5.87				
	5.2	35	+	6/6	707.20 ± 614.80	1:512	++	3/3	16.07 ± 2.49	1:256	++[α]	2/3	2.62 ± 2.31								
	5.3	32	+++	6/6	38294.17 ± 23816.82	1:512	+++	3/3	114.35 ± 34.34												
	5.4	37	+	6/6	781.48 ± 444.87[γ]	1:128	+	3/3	6.32 ± 5.64	1:256	++	3/3	2.81 ± 1.58								
Group 6 (uninfected)	6.1		NA	0/6	0.01	-	-	0/3	0.01	-	-	0/3	0.01								
	6.2		NA	0/6	0.01	-	-	0/3	0.01	-	-	0/3	0.01								
	6.3		NA	0/6	0.01	-	-	0/3	0.01												

[a] Day post-challenge when foetal death was detected by ultrasonography. The remaining foetuses lived until the end of the experiment.

[b] Placentomes in ewes that aborted and cotyledons in ewes that gave birth; "NA" samples from cotyledons were not evaluated for histopathology.

[c] Histopathological lesion severity: none detected/unrelated (-), mild lesions (+) (in placentome, 0-33.3% of the placentome showed lesions; in foetal brain, presence of local encephalitis), moderate lesions (++) (in placentome, 33.3-66.6% of the placentome showed lesions; in foetal brain, presence of diffuse encephalitis) and severe lesions of ovine neosporosis (+++) (in placentome, 66.6-99.9% of the placentome showed lesions; in foetal brain, presence of diffuse encephalitis and necrosis). * Autolysed; α No histopathological changes were found in one of the slides analysed.

[d] IFAT IgG antibody titres in foetal body fluids and in precolostral serum collected after birth in lambs born alive; "NA" not available.

[e] Fractions represent the number of positive samples by nested PCR/number of samples examined.

[f] Mean parasite load (tachyzoites/mg tissue) and standard deviation (SD). Considering that the *N. caninum* detection limit by real-time PCR is 0.1 parasites, negative samples (0 parasites) were represented as 0.01.

[β] Mummified foetuses were not evaluated for any of the parameters.

[γ] Samples from placental tissues exhibiting DNA degradation were excluded from parasite detection and/or parasite load analysis.

among challenge doses or routes of administration. Precolostral sera yielded positive titres ranging from 1:200 to 1:800 in all lambs born from infected groups. IFAT titre medians were 1:800 in G2 and G5 and 1:400 in G3 and G4, without significant differences among challenge doses (no statistical analysis was performed in G2 because only one lamb was born). Specific IgG responses against parasite antigen were not detected in lambs from the uninfected group (G6).

3.3. Pathology and lesion quantification

Placentas from aborting ewes showed multifocal non-purulent necrotic placentitis consisting of foci of necrosis in the placentomes mainly located in the caruncular septa. Several of these foci showed mineralization of the necrotic core area. No significant differences among challenge doses or routes of administration were found in the lesion severity in placentomes (Table 2).

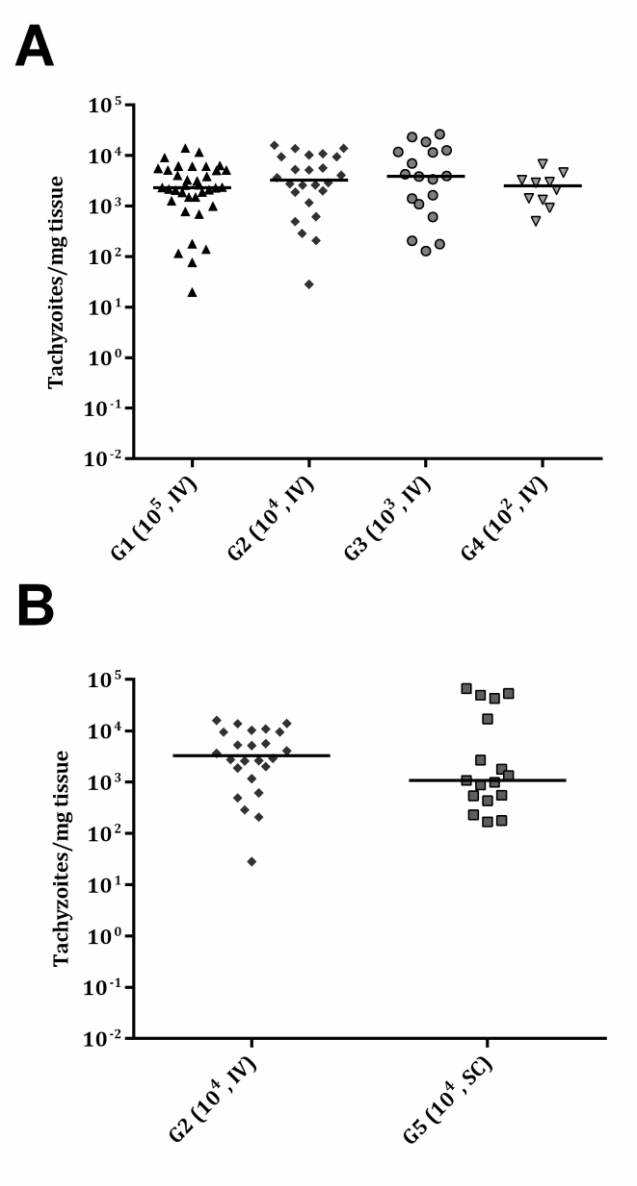
A multifocal nonpurulent necrotizing encephalitis characterized by the presence of randomly distributed glial foci surrounded by mononuclear cells was observed in foetal brains from all infected groups. Concerning challenge doses, lesion severity in the foetal brain was higher in G1 compared to those in G2 ($P < 0.05$), G3 ($P < 0.01$) and G4 ($P < 0.05$). By contrast, concerning routes of administration, no differences in lesion severity in the foetal brain were found between G2 and G5 (Table 2). Although aborted foetuses in G1 showed higher lesion severity in the foetal brain compared to those in G2 ($P < 0.05$), no significant differences in lesion severity in the foetal brain were found between lambs born in any of the infected groups or between aborted foetuses and lambs born in each group. No histopathological findings were found in the uninfected group (G6).

3.4. Parasite detection and burden in placental tissues and foetal brain

A few samples from placental tissues and foetal brain exhibited DNA degradation mainly due to mummification and were excluded from parasite detection and/or parasite load analysis (Table 2). *Neospora* DNA was detected in 100% of placentomes from ewes that aborted in G1 (36/36), G2 (24/24), G3 (18/18), G4 (12/12) and G5 (18/18), 100% of cotyledons from ewes that gave birth in G2 (4/4), G4 (12/12) and G5 (6/6) and 91.7% of cotyledons from ewes that gave birth in G3 (11/12) (Table 2). No significant differences in parasite detection in placentomes and cotyledons were found among challenge doses or between routes of administration. Analysing the mean parasite burden, measured as the number of tachyzoites per mg of tissue, no significant differences in parasite load in placentomes were found among challenge doses or between routes of administration (Figure 5A-B) (Table 2). Despite the low number of samples available, comparing challenge doses, parasite burden in cotyledons was significantly lower in G3 compared to those in G2 ($P < 0.01$) and G4 ($P < 0.001$) (Figure 6A). Likewise, comparing routes of administration, G2 showed a higher parasite load in cotyledons, with a trend towards significance compared to G5 ($P = 0.13$) (Figure 6B) (Table 2).

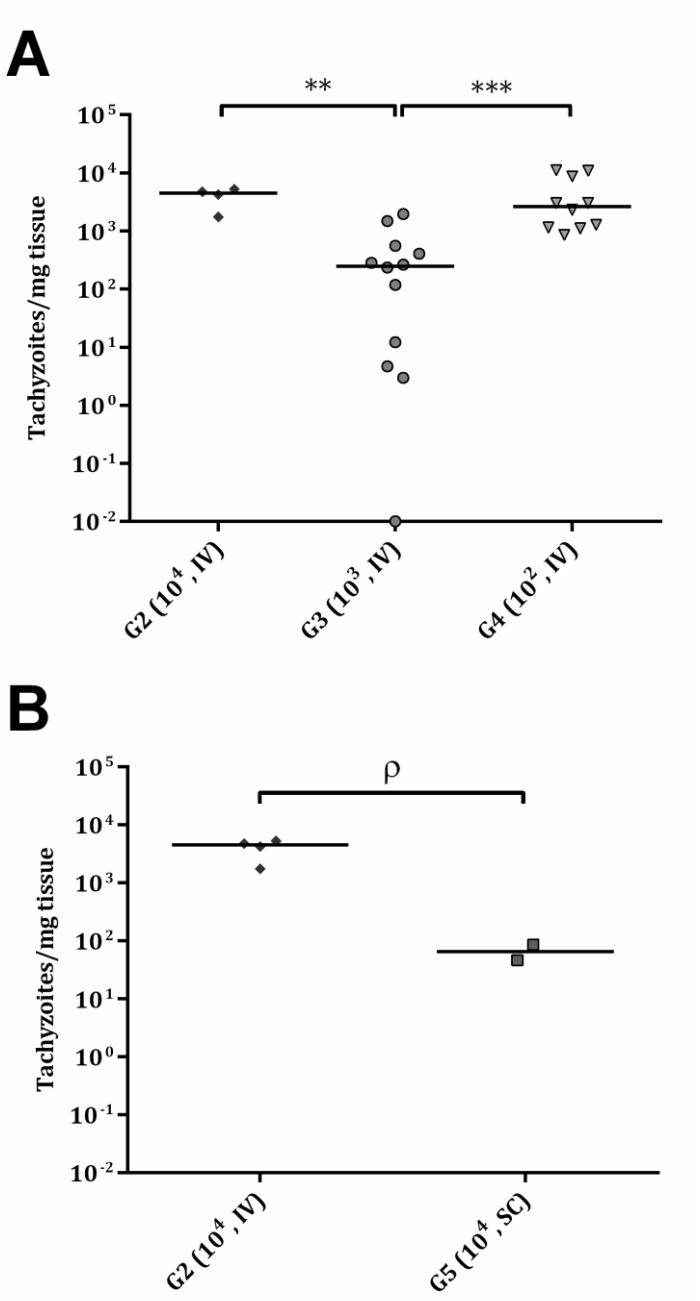
Parasite DNA in foetal brains was detected in 81.8% of samples in G1 (27/33), 91.7% in G2 (22/24), 66.7% in G3 (26/39), 90.5% in G4 (19/21) and 87.5% in G5 (21/24) (Table 2). Concerning challenge doses, significantly lower parasite detection was found in G3 compared to that in G2 ($P < 0.05$), and a trend towards significance was found compared to those in G1 ($P = 0.18$) and G4 ($P = 0.06$). Concerning routes of administration, no significant differences in parasite detection in the foetal brain were found between G2 and G5. Additionally, significantly lower parasite detection was found in aborted foetuses in G3 (12/18) than in aborted foetuses in G2 (20/21)

Figure 5 - Dot-plot graphs of *N. caninum* burdens in placentomes from intravenously challenged pregnant ewes (A) and pregnant ewes intravenously and subcutaneously challenged with 10^4 Nc-Spain7 tachyzoites and the uninfected group (B). Each dot represents individual values of parasite burden (number of parasites per mg of host tissue), and medians are represented as horizontal lines. Considering that the *N. caninum* detection limit by real-time PCR is 0.1 parasites, negative samples (0 parasites) were represented on the log scale as <0.1 (i.e., 10^{-2}). Parasite burdens were analysed using the non-parametric Kruskal–Wallis test followed by Dunn's test for comparisons between groups, as well as the Mann–Whitney test for pairwise comparisons.



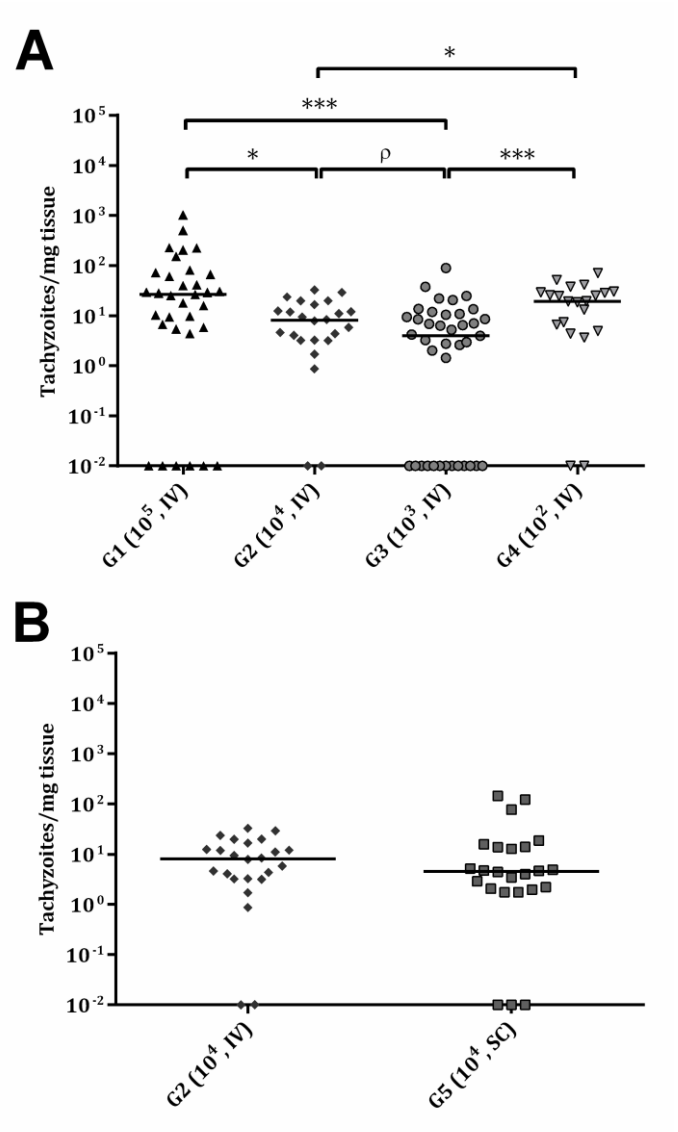
(P < 0.05), and a trend towards significance was found compared to that in G4 (12/12) (P = 0.05). Likewise, aborted foetuses from G1 (27/33) showed lower parasite detection compared to aborted foetuses from G4 (12/12) (P < 0.05). However, no significant differences in parasite detection were found among lambs born in any of the infected groups or among aborted foetuses or lambs born in each group. Parasite DNA was not detected in one aborted foetus in G1, in two aborted foetuses belonging to different dams in G3 (these three aborted foetuses were from twin pregnancies with foetal death and parasite detection in only one of the foetuses) or in one lamb in G3 (from a quadruplet pregnancy with parasite detection in

Figure 6 - Dot-plot graphs of *N. caninum* burdens in cotyledons from intravenously challenged pregnant ewes (A) and pregnant ewes intravenously and subcutaneously challenged with 10^4 Nc-Spain7 tachyzoites and the uninfected group (B). Each dot represents individual values of parasite burden (number of parasites per mg of host tissue), and medians are represented as horizontal lines. Considering that the *N. caninum* detection limit by real-time PCR is 0.1 parasites, negative samples (0 parasites) were represented on the log scale as <0.1 (i.e., 10^{-2}). Parasite burdens were analysed using the non-parametric Kruskal–Wallis test followed by Dunn's test for comparisons between groups, as well as the Mann–Whitney test for pairwise comparisons. (**) indicates $P < 0.01$, (***) indicates $P < 0.001$, and (ρ) indicates a trend towards significant differences among infected groups in each tissue.



three of the four lambs). Furthermore, when different challenge doses were compared, the parasite burden in the foetal brain was lower in G2 ($P < 0.05$) and G3 ($P < 0.001$) compared to that in G1 and G4. Likewise, G2 showed a higher parasite load in the foetal brain compared to G3, with a trend towards significance ($P = 0.06$) (Figure 7A). Nevertheless, when routes of administration were compared, no significant differences between parasite load in the foetal brain were found between G2 and G5 (Figure 7B) (Table 2).

Figure 7 - Dot-plot graphs of *N. caninum* burdens in foetal brain from intravenously challenged pregnant ewes (A) and pregnant ewes intravenously and subcutaneously challenged with 10^4 Nc-Spain7 tachyzoites and the uninfected group (B). Each dot represents individual values of parasite burden (number of parasites per mg of host tissue), and medians are represented as horizontal lines. Considering that the *N. caninum* detection limit by real-time PCR is 0.1 parasites, negative samples (0 parasites) were represented on the log scale as <0.1 (i.e., 10^{-2}). Parasite burdens were analysed using the non-parametric Kruskal–Wallis test followed by Dunn's test for comparisons between groups, as well as the Mann–Whitney test for pairwise comparisons. (*) indicates $P < 0.05$, (***) indicates $P < 0.001$, and (ρ) indicates a trend towards significant differences among infected groups in each tissue.



In addition, a lower parasite load in the foetal brain from aborted foetuses in G2 (G1, $P < 0.05$; G4, $P < 0.01$) and G3 (G1, $P < 0.05$; G4, $P < 0.01$) was found compared to that in G1 and G4. Additionally, a higher parasite load in the foetal brain from lambs born in G4 was found compared to that in G3, with a trend towards significance ($P = 0.13$). However, no significant differences in parasite load in the foetal brain were found between aborted foetuses or lambs in each group. As expected, all placental and foetal samples from G6 were negative.

4. Discussion

Ruminant challenge models are critical to the evaluation of vaccine and drug candidates to help tackle ruminant neosporosis and to study pathogenesis and host responses to infection (Benavides *et al.*, 2014). As an experimental animal model, sheep exhibit several advantages over cattle in terms of cost, space and infrastructure required, ease of handling of the animals and shorter duration of gestation. The pathogenesis of ovine neosporosis is not well known, and in contrast to the clinical outcome in cattle, infection during mid-pregnancy in sheep leads to abortion in most animals (McAllister *et al.*, 1996b; Buxton *et al.*, 1998; Weston *et al.*, 2009; Arranz-Solis *et al.*, 2015b). The impact of dose and route of challenge in abortion and vertical transmission of *N. caninum* in pregnant sheep has been studied little so far, and the results are difficult to compare. Recently, infection of 90-day-pregnant sheep with 10^6 tachyzoites of the Nc-Spain7 isolate caused abortion in all of them (Arranz-Solis *et al.*, 2015b; Sánchez-Sánchez *et al.*, 2018). Therefore, the aim of this study was to test different challenge doses and routes of administration in ewes infected at mid-term of gestation using the Nc-Spain7 isolate and establish an exogenous transplacental transmission model for ovine neosporosis that mimics natural *N. caninum* infections.

How the dose of tachyzoites equates to the ingestion of sporulated oocysts from definitive hosts and what level of environmental contamination is required to produce a similar outcome through natural exposure is not known (Weston *et al.*, 2009). It is possible that the doses of tachyzoites used in previous experiments (even up to 10^8) have been excessively aggressive for the infected sheep. This suggestion is supported by the fact that previous studies employing lower doses obtained more variable outcomes, ranging from a few aborted foetuses to the birth of weak or healthy lambs (McAllister *et al.*, 1996b; Weston *et al.*, 2009). Since intravenous infection of pregnant sheep using 10^6 tachyzoites of the Nc-Spain7 isolate at mid-gestation resulted in 100% abortion and parasite detection in the foetal brain in 83% of aborted foetuses (Arranz-Solis *et al.*, 2015b; Sánchez-Sánchez *et al.*, 2018), the challenge doses tested intravenously in the present study were less than the 10^6 tachyzoites previously assayed; tachyzoites were diluted 1:10 to a minimum concentration of 10^2 tachyzoites, similar to those evaluated by (Weston *et al.*, 2009). Subcutaneous inoculation closely mimics a natural primary infection as the parasite is ‘‘processed’’ through a draining lymph node before circulating in the blood (Dubey *et al.*, 2006). In cattle, it was reported that subcutaneous infection resulted in a foetal mortality that was 50% reduced compared to intravenous infection (Macaldowie *et al.*, 2004). Hence, in this study we aimed to investigate the outcome of *N. caninum* infection after subcutaneous inoculation in pregnant ewes using one of the intravenously tested doses. The dose of 10^4 tachyzoites was chosen for the subcutaneous administration since it is an intermediate-to-high dose between those tested by the intravenous route and also, because in a previous study, intravenous inoculation of 5×10^3 tachyzoites of New Zealand isolates at mid-gestation resulted in abortion in 50% of the ewes (Weston *et al.*, 2009).

With regard to clinical observations, foetal viability is the most significant parameter to be evaluated in an abortion model of neosporosis (Benavides *et al.*, 2014). Infection of cattle at mid-term gestation with 10^7 tachyzoites of the Nc-Spain7 isolate caused foetal death in 50% of infected animals after an experimental period of 6 weeks (Almería *et al.*, 2016). All pregnant ewes intravenously infected with 10^5 tachyzoites (G1) aborted in the same way as pregnant ewes intravenously infected with a 10-fold higher dose (10^6 tachyzoites) (Arranz-Solis *et al.*, 2015b; Sánchez-Sánchez *et al.*, 2018). The intravenous dose causing abortion in 50% of the infected animals was 10^2 tachyzoites (G4), and the foetal survival rate was higher compared to G1 (10^5, IV) with a trend towards significance possibly due to the lower number of animals in this group. It is remarkably lower than the intravenous dose of 5 x 10^3 causing abortion in 50% of the infected animals in the dose-titration study at mid-gestation using Nc-NZ1, Nc-NZ2 and Nc-NZ3 isolates (Weston *et al.*, 2009), suggesting higher virulence of Nc-Spain7 than New Zealand isolates in pregnant sheep. Likewise, intravenous infection at mid-gestation with 10^5 tachyzoites of the Nc-Spain7 isolate (G1) resulted in abortion of all pregnant ewes, whereas intravenous infection of pregnant ewes at mid-gestation with 1.7 x 10^5 tachyzoites of a mixture of the Nc-2 and Nc-Liverpool isolates caused abortion in 67% of the pregnant ewes (McAllister *et al.*, 1996b). However, because (McAllister *et al.*, 1996b) and (Weston *et al.*, 2009) used a mixture of different isolates within the same inoculum to assure infection, it is difficult to establish comparisons with these studies (Benavides *et al.*, 2014). The median number of abortion days in our study was similar to the time range of abortions in previous *N. caninum* experimental infections in pregnant sheep at mid-gestation (McAllister *et al.*, 1996a; Buxton *et al.*, 1998; Buxton *et al.*, 2001; Arranz-Solis *et al.*, 2015b). As reported by Weston *et al.* (2009) regarding the differences in the average time between abortion and parturition found after infection with decreasing doses, in the present study, the median survival times in G3 (10^3, IV) and more markedly in G4 (10^2, IV) were prolonged compared to those in G1 (10^5, IV), G2 (10^4, IV) and G5 (10^4, SC). In all aborting dams from G3 (10^3, IV) and G4 (10^2, IV), the coexistence at the time of euthanasia of live foetuses and dead foetuses in twin pregnancies suggests lower foetal damage in these groups because this observation was only found in one aborting dam in G1 (10^5, IV) and one aborting dam in G5 (10^4, SC). Mummified foetuses found in G1 (10^5, IV) and G2 (10^4, IV) have already been described after *N. caninum* experimental infection at mid-gestation in pregnant sheep (Buxton *et al.*, 1997). In G3 (10^3, IV), G4 (10^2, IV) and G5 (10^4, SC), pregnant ewes gave birth prematurely, similar to some pregnant ewes infected with 5 x 10^3 *N. caninum* tachyzoites (Weston *et al.*, 2009). Consequently, in G3 (10^3, IV), G4 (10^2, IV) and G5 (10^4, SC), stillborns and lambs died soon after birth showed a significant decrease in their bodyweight, as previously described Buxton *et al.* (1998). Likewise, a more significant decrease in lamb weight in G3 (10^3, IV) was found because it is known that with increasing litter size, the weight of the lambs is lower (Gardner *et al.*, 2007). The presence of a large number of stillborn lambs and weak lambs dying within 24 hours after birth could be explained by the absence of differences in parasite detection, parasite load and lesion severity from the foetal brain between aborted foetuses and lambs that gave birth in each group.

Another clinical parameter associated with infection is rectal temperature, and its increase is probably associated with the first replication cycles of the parasite in tissues and organs (Benavides *et al.*, 2014). Moreover, different temperature responses have been associated with the dose of parasite inoculums (Buxton *et al.*, 1997; Maley *et al.*, 2001; Maley *et al.*, 2003; Weston *et al.*, 2009). Intravenously challenged groups showed a unique fever peak after infection, although a dose-dependent

delay in the time of rectal temperature increase was found compared to the infection with 10^6 tachyzoites of the Nc-Spain7 isolate (Sánchez-Sánchez *et al.*, 2018), suggesting delayed parasite replication as lower infection doses were applied. However, as reported with 10^6 tachyzoites of the Nc-Spain7 isolate (Sánchez-Sánchez *et al.*, 2018), G1 (10^5, IV), G2 (10^4, IV) showed maximum rectal temperature on day 7 pi. Whereas the increase in rectal temperature persisted for 4 days in G1 (10^5, IV), G2 (10^4, IV) and G3 (10^3, IV), a less prolonged period with 3 days of rectal temperature increase was found in G4 (10^2, IV). Similar to different temperature responses found between intravenous and subcutaneous *N. caninum* challenge in cattle (Macaldowie *et al.*, 2004), after subcutaneous challenge in G5 (10^4, SC), a lower rectal temperature increase was found compared to intravenous challenge in G2 (10^4, IV). Likewise, a similar temperature response was found in G5 (10^4, SC) compared to the infection in sheep with the same dose (10^4 tachyzoites) of the Nc-Liverpool isolate (Buxton *et al.*, 1997). The biphasic temperature response found in the subcutaneous challenge group (G5) had been previously described after subcutaneous challenge in sheep (Buxton *et al.*, 1997; Buxton *et al.*, 1998; Buxton *et al.*, 2001) and cattle (Maley *et al.*, 2003; Benavides *et al.*, 2012). Previous studies in cattle have described differences in rectal temperatures between aborting and non-aborting dams (Almería *et al.*, 2010; Almería *et al.*, 2016) in the same way as observed in G4 (10^2, IV) in the present study.

Prefemoral lymph nodes were chosen as inoculation sites in G5 (10^4, SC) because they have been widely used for subcutaneous *N. caninum* challenge in cattle (Innes *et al.*, 2001b; Maley *et al.*, 2001; Maley *et al.*, 2003; Macaldowie *et al.*, 2004; Wiengcharoen *et al.*, 2011; Benavides *et al.*, 2012) and sheep (Buxton *et al.*, 1997; Buxton *et al.*, 1998; Buxton *et al.*, 2001). The clinical evaluation in G5 (10^4, SC) revealed enlargement of the left prefemoral lymph node as previously described Maley *et al.* (2001), Maley *et al.* (2003), Macaldowie *et al.* (2004) and Rocchi *et al.* (2011) after subcutaneous *N. caninum* challenge in cattle.

Intracellular protozoan parasites usually induce and are controlled by cellular immune responses. IFNγ plays a relevant role in controlling early *N. caninum* dissemination (Entrican, 2002; Hemphill *et al.*, 2006) and protecting against abortion in naturally infected cows (Lopez-Gatius *et al.*, 2007). Very short-lived IFNγ levels were produced in antigen-specific stimulation analyses at the end of the first and during the second week following infection with *N. caninum* and prior to mounting a specific IgG response. Similar IFNγ kinetics have been described in previous reports carried out in cattle upon stimulation of peripheral blood mononuclear cells (PBMCs) (Regidor-Cerrillo *et al.*, 2014) or in sheep serum (Arranz-Solís *et al.*, 2016). In G1 (10^5, IV), IFNγ released upon stimulation increased on day 7 pi in the same way that intravenous infection with 10^6 tachyzoites of the Nc-Spain7 isolate (Sánchez-Sánchez *et al.*, 2018). Nevertheless, the time course of IFNγ showed a delay until day 10 pi for IFNγ release after intravenous challenge in G2 (10^4, IV) and G3 (10^3, IV) and subcutaneous challenge in G5 (10^4, SC), although large individual variations were observed as previously described Rettigner et al. (2004b). Likewise, no significant increase in IFNγ was observed in G4 (10^2, IV) compared to the uninfected group (G6), which might have led to lower initial control of parasitaemia at the peripheral level, allowing a higher number of parasites to reach the placenta (Entrican, 2002; Innes, 2007). In fact, no differences in parasite load in the foetal brain between G4 (10^2, IV) and G1 (10^5, IV) could be due to the absence of a significant increase in IFNγ levels in pregnant ewes from G4 (10^2, IV) because a threshold IFNγ response is required to be beneficial against *N. caninum* (Almeria *et al.*, 2014; Almería and López-Gatius, 2015). Recently, it has been shown that the immune response appears to lead to superior priming of a cell-mediated immune

response in dams carrying live foetuses *versus* dams carrying dead foetuses (Bartley *et al.*, 2012; Darwich *et al.*, 2016). In this way, ewes that gave birth in G2 (10^4, IV) showed higher IFNγ levels on day 10 pi than those that aborted. Although no significant differences were found in IFNγ levels in G4 (10^2, IV) compared to the uninfected group (G6), a delay in the IFNγ peak was detected in ewes that gave birth. That, along with differences in rectal temperatures between aborting ewes and ewes that gave birth could suggest decreased early-stage replication of the parasite in ewes that gave birth in G4 (10^2, IV).

After intravenous infection of pregnant sheep at mid-gestation with 10^6 tachyzoites of the Nc-Spain7 isolate, IgG levels increased from day 12-14 pi (Arranz-Solís *et al.*, 2016; Sánchez-Sánchez *et al.*, 2018), whereas IgG levels increased from day 21 pi in G1 (10^5, IV). Furthermore, G1 (10^5, IV) exhibited higher IgG levels than those found with lower doses, possibly due to exposure to more abundant antigen and increased lymphoid stimulation similar to that reported by (Buxton *et al.*, 1997; Maley *et al.*, 2001; Bartley *et al.*, 2004; Weston *et al.*, 2009). In this study, all challenged animals showed seroconversion by ELISA, whereas the lower dose tested (50 tachyzoites of Nc-NZ1, Nc-NZ2 and Nc-NZ3 isolates) by (Weston *et al.*, 2009) revealed one seronegative animal at parturition by IFAT.

Concerning offspring, all aborted foetuses and lambs from the infected groups showed seropositive IFAT titres with no significant differences between challenge doses or routes of administration. Altogether, this finding indicates that once infection is established, it cannot be cleared from the host, and vertical transmission of the parasite occurred in all infected animals. In contrast, in a previous dose-titration study, none of the lambs and only 3 out of 5 lambs born from pregnant ewes intravenously infected with 50 and 5 x 10^3 *N. caninum* tachyzoites (Nc-NZ1, Nc-NZ2 and Nc-NZ3 isolates), respectively, were seropositive by IFAT (Weston *et al.*, 2009).

Because immune responses are not accurate enough to be used as indicators for disease or protection (Benavides *et al.*, 2014), parasite detection and quantification and histopathological assessment are essential. All placentomes from infected ewes were PCR positive, and no significant differences in parasite load or lesion severity were found, so immune responses were unsuccessful in preventing the colonization and multiplication of *N. caninum* in the placentomes of aborting ewes. No difference was found in parasite detection between placentomes from intravenously infected ewes at mid-gestation with 10^6 tachyzoites of the Nc-Spain7 isolate (PCR-positive samples from 83% to 100%) (Arranz-Solis *et al.*, 2015b; Sánchez-Sánchez *et al.*, 2018) and placentomes from intravenously infected aborting ewes in this study. *N. caninum* DNA was also widely detected in cotyledons from ewes that gave birth with no significant differences in parasite detection, similar to that reported by (Weston *et al.*, 2009), which found all PCR-positive cotyledons in ewes infected with 5 x 10^3 *N. caninum* tachyzoites. However, lower parasite loads were found in cotyledons from ewes that gave birth in G3 (10^3, IV), which, along with lower IFNγ levels detected, could suggest mild infection in these animals. When routes of administration were compared, no significant differences were identified in parasite detection, parasite load or lesion severity in placentomes, however, cotyledons from the subcutaneously infected group (G5) showed lower parasite burden compared to G2 (10^4, IV) with a trend towards significance, maybe influenced by the lower number of animals in this group.

In transplacental transmission models for ruminant neosporosis, it is crucial to evaluate parasite presence, parasite load and lesions in foetal tissues (Benavides *et al.*, 2014). The central nervous system has been described as

the target tissue in foetuses from *N. caninum* infection of cattle (Almería *et al.*, 2016) and sheep (Arranz-Solis *et al.*, 2015b) at mid-pregnancy. Concerning infective doses, detection percentages in foetal brains from G1 (10^5, IV), G2 (10^4, IV) and G4 (10^2, IV) showed no significant differences among them, nor when they were compared to detection in foetal brains after intravenous infection of pregnant ewes with 10^6 tachyzoites of the Nc-Spain7 isolate at mid-gestation (94% of PCR-positive samples) (Sánchez-Sánchez *et al.*, 2018). These results are not in accordance with differences in the proportion of positive brains in foetuses/lambs of a dose-titration study in pregnant sheep at mid-gestation using other isolates (Weston *et al.*, 2009). Conversely, the lower detection percentage in foetal brains from G3 (10^3, IV) could be because of the higher number of foetuses per dam in this group; fewer parasites that cross the placental barrier reach each foetus. Brain-negative foetuses arising from multiple pregnancies have already been reported in ewes infected at mid-gestation with 10^6 tachyzoites of Nc-Spain7 (Arranz-Solis *et al.*, 2015b). Foetuses and lamb showing PCR-negative brains in this study were seropositive by IFAT in the same way as in (Porto *et al.*, 2016), and brain lesions were identified, suggesting the presence of very low parasite load in their brain.

We hereby describe the outcome of *N. caninum* infection in pregnant sheep at mid-gestation by performing experimental infections using different numbers of tachyzoites of the virulent Nc-Spain7 isolate and different routes of inoculation. Intravenous infection with 10^5 tachyzoites was sufficient to trigger 100% abortion in the same way as 10^6 tachyzoites previously assayed. In addition, intravenous infection with 10^5 tachyzoites showed distinct immune responses and parasite load in the foetal brain. Surprisingly, the differences between the highest and the lowest intravenous doses were much smaller than expected, and we here demonstrate that experimental infection with as few as 100 tachyzoites could induce abortion in 50% of the ewes, and parasite load in the foetal brain was similar to that with the highest dose. Regarding the routes of inoculation, subcutaneous infection with 10^4 tachyzoites showed similar abortion rates and vertical transmission to intravenous infection.

In conclusion, with the doses and routes of administration evaluated, we propose that future studies using an abortion model for ovine neosporosis should be carried out using the intravenous route of administration and a challenge dose of 10^5 tachyzoites (100% abortion and vertical transmission), which will then allow to obtain more accurate and realistic conclusions in studies testing vaccine and drug candidates. However, further studies are necessary to evaluate the outcome of infection with 10^5 tachyzoites by the subcutaneous route of administration.

Competing interests

The authors declare that they have no competing interests.

Author´s contributions

IF, JB, JRC and LMO conceived the study and participated in its design. RSS wrote the manuscript, with results interpretation and discussion inputs from IF, MPD and LMO. LMF and TN selected the animals and carried out the reproductive programme. JRC prepared the inocula and carried out the infections. RSS, MR, JBM, MPD, MGH, ET and JB participated in inoculation, clinical examination and sampling of animals and performed necropsies and histopathological analyses. RSS performed PCR and qPCR analysis, serological assays, statistical analysis and interpreted the results. All authors read and approved the final manuscript.

Acknowledgements

We gratefully acknowledge Neus Callizo Esteve from Clinical Veterinary Hospital (Complutense University of Madrid, Spain) and José María González (University of Zaragoza, Spain) for their excellent technical assistance. The Animal Experimentation Service (SEA) at the University of Zaragoza is acknowledged for using their facilities to carry out the reproduction programme. Roberto Sánchez Sánchez is supported by a fellowship from the Spanish Ministry of Education, Culture and Sports (MECD) as a part of the Programme of Training of University Teaching Staff (FPU, grant number FPU13/03438). This work was supported by the Ministry of Economy and Competitiveness (AGL2016-75935-C2) and Community of Madrid, Spain (PLATESA, S2013/ABI2906).

Objetivo 3

Evaluación de la seguridad y eficacia de los compuestos inhibidores de la CPDK1 (BKI) frente a *T. gondii* y *N. caninum* en ovejas gestantes

T. gondii y *N. caninum* son parasitos apicomplejos muy relacionados filogenéticamente y considerados como unos de los principales agentes causantes de aborto infeccioso en pequeños rumiantes y ganado vacuno respectivamente. Actualmente no hay fármacos disponibles en el mercado para el tratamiento de la toxoplamosis y neosporosis en rumiantes. Los inhibidores de proteinas quinasas que compiten por el sitio de unión del ATP en la proteína quinasa dependiente de calcio tipo 1 (CDPK1) de los parásitos apicomplejos (BKIs) han mostrado ser altamente eficaces *in vitro* y en modelos de animales de laboratorio frente a numerosos parásitos apicomplejos. Para la consecución de este objetivo las ovejas gestantes se dosificaron por vía oral con el BKI-1294 (5 dosis de 100 mg/kg cada 48 horas) o por vía subcutánea con el BKI-1553 (con dos pautas de dosificación: 1ª dosis de 35 mg/kg y una semana después una 2ª dosis de 10 mg/kg ó 7 dosis de 10 mg/kg cada 48 horas). Se evaluó la eficacia de estos fármacos administrados a las 48 horas de la infección por vía oral con ooquistes del aislado TgShSp1 (BKI-1294) o por vía intravenosa con taquizoitos del aislado Nc-Spain7 (BKI-1553). La seguridad de estos fármacos se evaluó mediante la monitorización de las temperaturas rectales y la viabilidad fetal, el estudio de los parámetros hematológicos y bioquímicos asi como mediante la observación de la consistencia fecal (tras la administración oral) o de las reacciones locales adversas (tras la administración subcutánea). La eficacia de los inhibidores de la CDPK1 fue evaluada mediante la monitorización clínica, el estudio de la respuesta inmune y de las lesiones y la determinación de la presencia y carga de los parásitos en placenta y/o tejidos fetales diana. Además, fueron determinados los niveles plasmáticos del BKI-1294 y BKI-1553 en ovejas asi como los del BKI-1553, tras la administración de una única dosis de 10 mg/kg, tanto en los fetos como en las ovejas. Tras la administración subcutánea del BKI-1553 y oral del BKI-1294 las ovejas gestantes presentan niveles terapéuticos de fármaco en plasma con concentraciones máximas de 11 μM y 2 μM y mínimas de 4 μM y 0.4 μM para el BKI-1553 y BKI-1294 respectivamente. Además, el BKI-1553 fue capaz de atravesar la barrera placentaria ya que se encontraron niveles terapéuticos en plasma fetal de 1 μM. Sin embargo, el BKI-1553 y de forma más acusada el BKI-1294 presentaron un rápido aclaramiento plasmático. En relación a la seguridad, tanto el BKI-1294 como el BKI-1553 parecen seguros ya que no se observaron alteraciones en la temperatura, en los parámetros hematológicos y bioquímicos ni en la gestación o daño local asociado con estos fármacos. Sin embargo, se observó diferente seguridad para cada una de las rutas de administración utilizadas. Mientras que la administración oral no incrementó la temperatura rectal ni modificó la consistencia fecal, la administración subcutánea desencadenó la formación de nódulos dérmicos asociados a un incremento de temperatura y monocitosis.

La administración del BKI-1294 a ovejas infectadas con *T. gondii* o del BKI-1553 a ovejas infectadas con *N. caninum* hizo que fuera menor el incremento de las temperaturas asociado a la infección probablemente debido a una menor replicación de los parásitos. Los estudios *in vitro* han puesto de manifiesto la presencia de complejos intracelulares multinucleados inducidos por el BKI-1294 y BKI-1553, con una sobreexpresión del antigeno de taquizoitos SAG1 y del marcador de bradizoito SAG1. Estos complejos multinucleados se componen de multiples prezoitos incapaces de separarse y formar taquizoitos y podrían influir sobre la respuesta inmune celular y humoral frente a

T. gondii y *N. caninum* ya que se la administración de estos fármacos desencadena un incremento de la producción de IFNγ y la presencia de anticuerpos anti-*T. gondii* SAG1. En modelos ovinos gestantes en los cuales todas las ovejas sin tratar abortaron, el tratamiento con el BKI-1294 en ovejas infectadas con *T. gondii* desencadenó el nacimiento de un 76% de corderos sanos y el tratamiento con el BKI-1553 en ovejas infectadas con *N. caninum* confirió una protección del 37-50% frente al aborto. En relación a la transmisión vertical, mientras que el BKI-1553 no protegió frente a la transmisión vertical de *N. caninum*, aunque disminuyó los efectos de la infección reduciendo las lesiones y la presencia del parásito y la carga en cerebro fetal, el BKI-1294 fue capaz de prevenir la transmisión vertical de *T. gondii* en el 53% de los corderos.

Bumped kinase inhibitor BKI-1294 is safe and produces high rates of protection against abortion and vertical transmission in sheep experimentally infected with *Toxoplasma gondii* during pregnancy

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To be submitted to Journal of Antimicrobial Chemotherapy

Abstract

Toxoplasma gondii is one of the main abortifacient in sheep, causing important economic losses. Previous studies on drug efficacy using monensin, folate inhibitors and decoquinate showed moderate protection against abortion and vertical transmission of *T. gondii* in pregnant sheep. Although a live-attenuated vaccine is available to protect against abortion in *T. gondii* infected sheep, at present no labelled drugs are marketed. Bumped kinase inhibitors (BKIs), which are ATP-competitive inhibitors of calcium dependent protein kinase 1 (CDPK1), showed to be highly efficacious against several apicomplexan parasites *in vitro* and in laboratory animal models. We present here the pharmacokinetics, safety and efficacy of 5 doses at 100 mg/kg orally of BKI-1294 in a pregnant sheep model of *T. gondii* infection. BKI-1294 showed systemic exposure in pregnant ewes, with maximum concentrations of 2 μM and trough concentrations of 0.4 μM at 48 hours after each dose. Oral administration of BKI-1294 in sheep at mid-pregnancy wa safe since no variation on fecal consistency, rectal temperatures and haematological and biochemical parameters or abortions were found. In ewes infected with a *T. gondii* oocyst dose 100% lethal for the foetuses, BKI-1294 treatment resulted in a decrease on rectal temperatures upon infection, a strong IFNγ production, a low humoral immune response to soluble tachyzoite antigens but high levels of anti-SAG1 antibodies and a decrease of 76% in the perinatal mortality. In addition, none of the lambs born alive showed lesions and vertical transmission was prevented in 53% of them. These results provide a proof of concept for the therapeutic use of BKIs in ovine toxoplasmosis.

Keywords: *Toxoplasma gondii*; sheep; treatment; pregnancy; abortion; vertical transmission; protein kinase inhibitor; BKI-1294

1. Introduction

Toxoplasma gondii is an apicomplexan parasite, being one of the main abortifacient in sheep and, therefore causing important economic losses due to abortions after primo-infection of pregnant sheep (Dubey, 2010). Congenital transmission of *T. gondii* mainly occurs after primoinfection, through ingestion of oocysts during pregnancy (Innes *et al.*, 2009). Infection during the early and mid-pregnancy is usually associated with the occurrence of abortion, while infection in late pregnancy would produce a congenitally infected but viable lamb (Buxton *et al.*, 2007). Once the infection occurs, there is a generally delay of 4 weeks until the occurrence of the abortion (Dubey, 2010). However, in a number of experimental studies, oral inoculation of sheep with sporulated oocysts resulted in earlier abortion between days 7 and 14 pi (Trees *et al.*, 1989; Owen *et al.*, 1998b; Castaño *et al.*, 2014; Castaño *et al.*, 2016).

For the control of ovine toxoplasmosis, different measures have been suggested; however, the combination of different approaches is known to be the optimal strategy (Dubey, 2009b). The implementation of farm biosecurity protocols, hygienic measures and management practices should be adopted in all farms, for reducing the level of environmental contamination with *T. gondii* oocysts via cat faeces. Despite being properly designed and meticulously practised, globally, these control measures alone are not cost-viable or completely effective in eliminating toxoplasmosis from a flock, and it is necessary to complement them with an immune-chemotherapeutical approach (Sánchez-Sánchez et al., accepted for publication). To date, the control of ovine toxoplasmosis is primarily based on preventing its horizontal

transmission and on the establishment of a vaccination programme with a live attenuated S48 strain (Toxovax™, MSD) (Buxton *et al.*, 1991; Buxton *et al.*, 1993b). The benefits are associated with protection against abortions induced by *T. gondii* during pregnancy and a decrease in tissue cyst development (Innes *et al.*, 2009). Although experimental studies have revealed effectiveness of several drugs *in vitro* and in laboratory animal models (Sánchez-Sánchez et al., accepted for publication), only monensin (Buxton *et al.*, 1987; Buxton *et al.*, 1988), folate inhibitors (Buxton *et al.*, 1993a) and decoquinate (Buxton *et al.*, 1996) have been evaluated against *T. gondii* in pregnant sheep. In these studies, protection against abortion was found in 20-40% of infected ewes (Buxton *et al.*, 1988; Buxton *et al.*, 1996), however there is limited or no protection against vertical transmission (Buxton *et al.*, 1987; Buxton *et al.*, 1988; Buxton *et al.*, 1993a; Buxton *et al.*, 1996). Therefore, currently there is no a labelled drug available against ovine toxoplasmosis in the market.

Anti-parasitic drug development based on targeting kinase enzymes is a well-established approach (Rotella, 2012). Calcium dependent protein kinase 1 (CDPK1) represents a promising drug target, as CDPK1 is encoded by the apicoplast DNA, and is thus absent from mammalian hosts (Lourido *et al.*, 2010; Murphy *et al.*, 2010; Ojo *et al.*, 2010; Cardew *et al.*, 2018).

CDPK1 activity is essential for microneme secretion, host cell invasion, and egress of *T. gondii* (Kieschnick *et al.*, 2001; Lourido *et al.*, 2010; Lourido *et al.*, 2012) and can be effectively targeted by a class of ATP-competitive compounds, collectively named bumped kinase inhibitors (BKIs). BKIs have a broad-spectrum activity that affects many apicomplexan parasites (Van Voorhis *et al.*, 2017). BKI-1294 was effective against *T. gondii in vitro* (Winzer *et al.*, 2015), and *in vivo* against acute (Lourido *et al.*, 2013; Doggett *et al.*, 2014) and chronic toxoplasmosis in mice (Lourido *et al.*, 2013) as well as against vertical transmission in a pregnant mice model of toxoplasmosis (Müller *et al.*, 2017c). We report here on the safety and efficacy of BKI-1294 treatment in pregnant sheep experimentally infected with *T. gondii* oocysts at mid-gestation.

2. Materials and methods

2.1. Ethics statement

All protocols involving animals were approved by the Animal Welfare Committee of the Community of Madrid, Spain (PROEX 166/14), following proceedings described in Spanish and EU legislation (Law 32/2007, R.D. 53/2013, and Council Directive 2010/63/EU). All animals used in this study were handled in strict accordance with good clinical practices, and all efforts were made to minimize suffering.

2.2. Animals and experimental design

Thirty-five pure Rasa Aragonesa breed female ewes aged 12 months were selected from a commercial flock. All animals were seronegative for *T. gondii*, *N. caninum*, Border disease virus (BDV), Schmallenberg virus (SBV), *Coxiella burnetii* and *Chlamydia abortus* as determined by enzyme linked immunosorbent assay (ELISA). They were oestrus-synchronized and mated with pure-breed Rasa Aragonesa tups for 2 days. Pregnancy and foetal viability were confirmed by ultrasound scanning (US) on day 40 post-mating, and twenty-five pregnant sheep were selected for the experiment. Pregnant ewes (n = 25) were randomly distributed into four experimental groups (see Table 1) and housed at the animal facilities of the Animal Health Department in the Faculty of Veterinary Sciences (Complutense University of Madrid, Spain). Fifteen ewes were allocated into groups 1 (G1; n=7) and 2 (G2; n=8), which were dosed orally with 1000 *T. gondii* sporulated oocysts of the type II (genotype #3) *T. gondii* isolate TgShSp1 (Sánchez-Sánchez et al., submitted) at day 90 of gestation (dg). The ten remaining

pregnant ewes were allocated to groups 3 (G3; n=5) and 4 (G4; n=5), which received 50 mL of phosphate-buffered saline (PBS) at 90 dg.

BKI-1294 was synthesized by WuXi and further purified in the Department of Chemistry of the University of Washington. The drug formulation was prepared by dissolving the compound in 60% PHOSAL® 53 MCT (LIPOID GmbH, Ludwigshafen, Germany), 30% Polyethyleneglycol 400 (PEG-400) (Sigma-Aldrich, Madrid, Spain) and 10% Ethanol 96º (Panreac, Barcelona, Spain) by heating at 37°C and shaking for 3 hours at a final concentration of 45 mg/ml. Starting at 48 hours post-infection (pi), BKI-1294 was administered orally through an oro-ruminal probe to G1 and G3, both groups at 100 mg/kg bodyweight, 5 doses every other day. Each ewe from G1 and G3 received 146.16 ± 20.41 mL for each dose. In addition, ewes from G4 received 5 doses (139.79 ± 7.76 mL), every other day, of vehicle alone. Ewes from G3 (non-infected, treated) and G4 (non-infected, vehicle alone) were kept alive until the end of the experiment. Safety of BKI-1294 was evaluated by clinical monitoring of rectal temperatures, fecal consistency and foetal viability and evaluation of haematological and biochemical parameters. Efficacy of this compound against congenital toxoplasmosis in sheep was assessed by the presence of perinatal mortality (foetal death during pregnancy and stillbirths) and vertical transmission in live lambs (seropositivity and parasite detection/lesion in brain or lungs) according to Sánchez-Sanchez et al. (submitted).

2.3. Clinical monitoring

Pregnant ewes were observed daily throughout the entire experimental period. Foetal viability was assessed post-infection by US monitoring of foetal heartbeat and movements once a week. Rectal temperatures were recorded daily from day 0 until 14 days pi and then weekly. Animals were considered febrile when the rectal temperatures was over 40°C (Diffay *et al*., 2002).

When foetal death occurred, or 48 hours after parturition, dams and lambs were first sedated with xylazine (Rompun, Bayer, Mannhein, Germany) and then euthanized by an intravenous overdose of embutramide and mebezonium iodide (T61, Intervet, Salamanca, Spain). Deliveries up to day 140 of pregnancy were considered premature. Lambs born from day 141 onwards were weighed immediately after birth. Lambs born alive were clinically inspected for 48 hours after birth.

2.4. Collection of blood samples

Blood samples to evaluate peripheral immune responses were collected from G1 and G2 (both infected) and G4 (uninfected/vehicle alone) prior to infection, at 3, 5, 7 and 10 days pi and then weekly by jugular venipuncture into 5 mL vacutainer tubes (Becton Dickinson and Company, Plymouth, UK) with and without lithium heparin as anticoagulant.

In addition, in G3 (uninfected/BKI-1294 treated) and G4 (uninfected/vehicle alone) haematological and biochemical parameters before (day 0 pi) and after treatment (4 days after the 5th BKI-1294 dose) were assessed in blood samples collected into 5 mL vacutainer tubes (Becton Dickinson and Company, Plymouth, UK), with ethylenediaminetetraacetic acid (EDTA) as anticoagulant and into 5 mL vacutainer tubes (Becton Dickinson and Company, Plymouth, UK) without anticoagulant. Tubes without

Table 1 - Experimental design

Group	Number of pregnant ewes	Number of foetuses/lambs	Challenge (P.O.)	Treatment (P.O.)
G1	7	17	1000 TgShSp1 sporulated oocysts	BKI-1294, 5 doses at 100 mg/kg bodyweight every other day
G2	8	17	1000 TgShSp1 sporulated oocysts	None
G3	5	8	PBS	BKI-1294, 5 doses at 100 mg/kg bodyweight every other day
G4	5	7	PBS	Vehicle alone, 5 doses every other day

P.O.: *per os*, orally

anticoagulant were allowed to clot and were centrifuged to obtain serum samples that were stored at −80°C until analysis.

To determine BKI-1294 exposure, blood samples from the treated groups G1-G3 were collected at multiple time points by jugular venipuncture into 1 mL tubes (Aquisel, Barcelona, Spain) containing lithium heparin. From G1 and G3, blood was collected: prior to BKI-1294 administration; 1, 2, 4, 8, 12, 24, 30 and 48 hours after the 1st dose; 8, 12, 24, 30 and 48 hours after the 2nd, 3th, 4th and 5th doses; and finally, daily until 7 days after the 5th dose. Heparinised blood samples were centrifuged at 805 x *g* for 30 min at 4°C, and plasma samples were stored at -20°C until analysis by liquid chromatography tandem mass spectrometry (LCMS/MS).

Precolostral serum was collected from lambs born in G1, G2 and G4 and maintained at −80°C for subsequent serological analysis. To avoid any accidental suckling from lambs born overnight, udders were covered with a piece of cloth 1 week before the expected date of delivery as a preventive measure.

2.5. *Post-mortem* collection of tissue and body fluid samples

To evaluate vertical transmission of the parasite, brains and lungs from foetuses in G1, G2 and G4 were stored at −80°C for DNA extraction and fixed in 10% formalin for histopathological examination. Foetal thoracic and abdominal fluids were also collected from foetuses in G1, G2 and G4 and maintained at −80°C for serology.

2.6. BKI-1294 pharmacokinetics

BKI-1294 was extracted from the plasma samples taken from groups G1 and G3 using acetonitrile / 0.1% formic acid with an internal standard. A standard curve was prepared for comparison and quantification. BKI-1294 was measured with an Acquity ultra performance liquid chromatography (UPLC) system in tandem with a Xevo TQ-S mass spectrometer (Waters, Milford, MA, USA). Calculations of maximum concentration (C_{max}) for each dose, and area-under-the-curve (AUC) were determined using Prism (GraphPad, San Diego, CA).

2.7. Haematological and biochemical analyses

As previously described Sánchez-Sánchez *et al.* (2018), complete blood counts (CBCs) were determined in whole blood using the automated laser-based haematology analyser Advia 120 (Siemens, Healthcare Diagnostics GmbH, Eschborn, Germany). Biochemical parameters were measured in serum using the sequential automatic autoanalyzer Konelab 30 (Thermo Fisher Scientific, Waltham, USA). Ions were assessed in serum using a Microlyte 3 (Beckman Coulter, Brea, USA). Reference values were obtained from Antón and Mayayo (2007).

2.8. Peripheral blood cell stimulation assay and assessment of interferon-gamma (IFNγ) production

Peripheral blood stimulation assay and assessment of IFNγ production were carried out as previously described Sánchez-Sánchez *et al.* (2018), but using *T. gondii* soluble antigen for stimulation.

2.9. Serological analyses: ELISA and IFAT

T. gondii-specific IgG antibody levels in sheep were measured using an in-house indirect ELISA similarly as previously described Castaño *et al.* (2014). 96-well microtiter plates (Thermo Fisher Scientific, Waltham, USA) were coated with 100 µL soluble *T. gondii* antigen (1.5 µg/mL in 100 mM carbonate buffer pH 9.6) overnight at 4°C. Plates were blocked and serum samples were diluted 1:100 using 3% bovine serum albumin diluted in PBS containing 0.05% Tween 20 (PBS-T). Subsequently, horseradish peroxidase-conjugated protein G (Sigma-Aldrich, Madrid, Spain) diluted 1:6000 in PBS-T was added and after that, ABTS (Roche, Basilea, Switzerland) was used as substrate. The reaction was stopped by adding 100 µL of 0.3 M oxalic acid and the optical density (OD) was read at 405 nm (OD405). For each plate, values of the OD were converted into a relative index percent (RIPC) using the following formula: RIPC = (OD405 sample – OD405 negative control)/(OD405 positive control–OD405 negative control) × 100. A RIPC value ≥10 indicates a positive result.

In addition, anti-*T. gondii* SAG1 (p30) antibodies were measured using a commercial ELISA "ID Screen Toxoplasmosis Indirect Multi species" (IDvet, Grabels, France). Following manufacturer´s instructions, for each sample, values of the OD were converted into the S/P percentage (S/P %) using the following formula: S/P % = (OD450 sample – OD450 negative control)/(OD450 positive control–OD450 negative control) × 100. A S/P % ≥50 indicates a positive result.

Indirect fluorescent antibody test (IFAT) was used to detect specific IgG anti-*Toxoplasma* antibodies in foetal fluids and precolostral sera, adapting the technique previously described for IFAT analysis in *N. caninum* infected animals (Alvarez-Garcia *et al.*, 2003), using an anti-sheep IgG (Sigma-Aldrich), diluted 1:200 in Evans Blue (Sigma-Aldrich). Foetal fluids and precolostral sera were diluted at two-fold serial dilutions in PBS starting at 1:8 (for foetal fluids) and 1:50 (for precolostral sera) up to the endpoint titre. Continuous tachyzoite membrane fluorescence at a titre ≥ 8 for foetal fluids or ≥ 50 for precolostral sera was considered a positive reaction.

2.10. Histopathology

After fixation for five days, brains and lungs from foetuses were cut coronally, embedded in paraffin wax and processed by standard procedures for haematoxylin and eosin (HE) staining. Conventional histological evaluation was carried out on all sections.

2.11. DNA extraction and PCR for parasite detection

Genomic DNA was extracted from 50–100 mg of brain and lung samples from foetuses using the commercial Maxwell® 16 Mouse Tail DNA Purification Kit, developed for the automated Maxwell® 16 System (Promega, Wisconsin, USA), following the manufacturer's recommendations. The concentration of DNA for all samples was determined by spectrophotometry and adjusted to 50–100 ng/µL. *T. gondii* DNA detection was carried out by an ITS-1 PCR adapted to a single tube as previously described Castaño *et al.* (2014). Samples from the group receiving vehicle alone (G4) were included in each round of DNA extraction and PCR as negative controls.

2.12. Statistical analysis

Occurrence of perinatal mortality in ewes was analysed by the Kaplan–Meier survival method and perinatal survival curves were then compared by the Log-rank (Mantel-Cox) test. Comparison of the number of foetuses/lambs suffering perinatal mortality was done using the $\chi 2$ or Fisher's exact F-test. Rectal temperatures were analysed using One-way ANOVA followed by Tukey's multiple test until 14 days pi. Cellular immune responses were analysed using Two-way ANOVA of repeated measures until day 7 pi. Weights of the lambs were compared using the non-parametric Kruskal–Wallis test followed by Dunn's test for comparisons between groups, as well as the Mann–Whitney test for pairwise comparisons. C_{max} and AUC for ewes infected (G1) and uninfected (G3) as well as within G1 for ewes suffering perinatal mortality *vs* ewes that gave birth and for ewes with at least one positive lamb *vs* those ewes with no positive offspring were evaluated using the Mann–Whitney test for pairwise comparisons. Statistical significance for all analyses was established at $P < 0.05$. All statistical analyses were performed using GraphPad Prism 6.01 software (San Diego, CA, USA).

3. Results

3.1. Pharmacokinetics

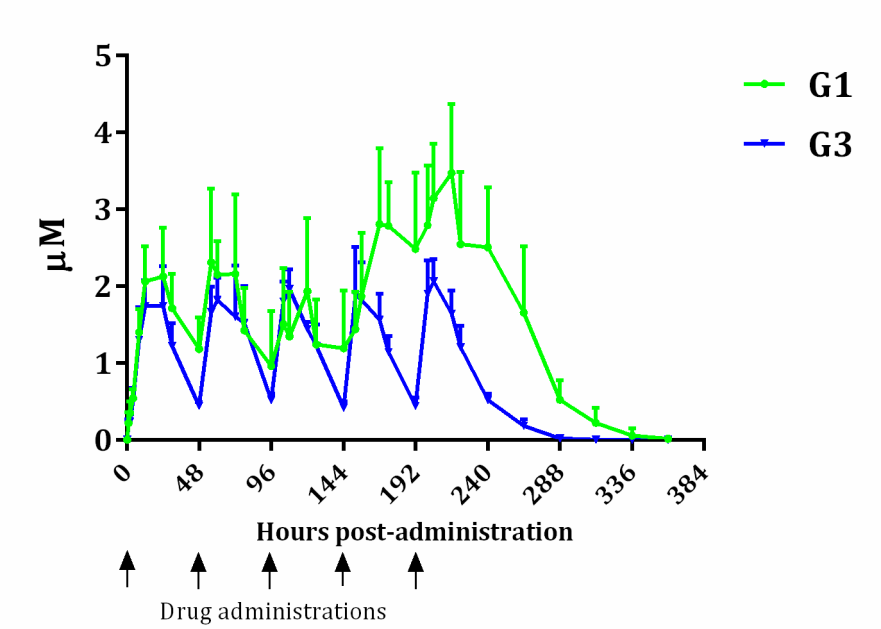
In G1, at 8-24 hours after BKI-1294 administration, C_{max} of 2.2 ± 0.8 µM after the 1st, 2nd and 3rd doses and of 3.5 ± 0.7 µM after the 4th and 5th doses were reached. Likewise, trough plasma concentrations of 1.1 ± 0.6 µM 48 hours after the 1st, 2nd and 3rd doses and of 2.4 ± 0.8 µM 48 hours after the 4th and 5th doses, and detectable drug levels until 6-7 days after the last dose were found (Figure 1). In G3, C_{max} of 2 ± 0.3 µM were reached 8-24 hours after each dose, with trough plasma concentrations of 0.4 ± 0.1 µM 48 hours after 1st, 2nd, 3rd, 4th and 5th dose and detectable drug levels until 3-4 days after the last dose (Figure 1).

There were significant differences observed between the AUCs ($P < 0.05$) and C_{max} at both the 4th ($P < 0.05$) and 5th ($P < 0.05$) doses for the infected sheep in G1 and the uninfected sheep in G3. No significant differences were observed for the C_{max} or AUCs between the ewes that suffered perinatal mortality and those that gave birth in G1. There were also no significant differences for C_{max} or AUC for the ewes from G1 with at least one positive lamb compared to those ewes with no positive offspring.

3.2. Clinical observations

In sheep that remained uninfected but received BKI-1294 treatment (G3) or vehicle alone (G4), when analysing the recorded rectal temperatures, no significant increase was found throughout 12 days after treatment (Figure 2). In addition, no alterations on the faecal consistency was found. Likewise, no perinatal mortality was detected in uninfected but BKI-

Figure 1 - BKI-1294 plasma concentrations in infected and uninfected ewes. Values from infected ewes (G1) and uninfected ewes (G3) dosed with BKI-1294. Each point represents the mean + S.D. at the different sampling times for each group.



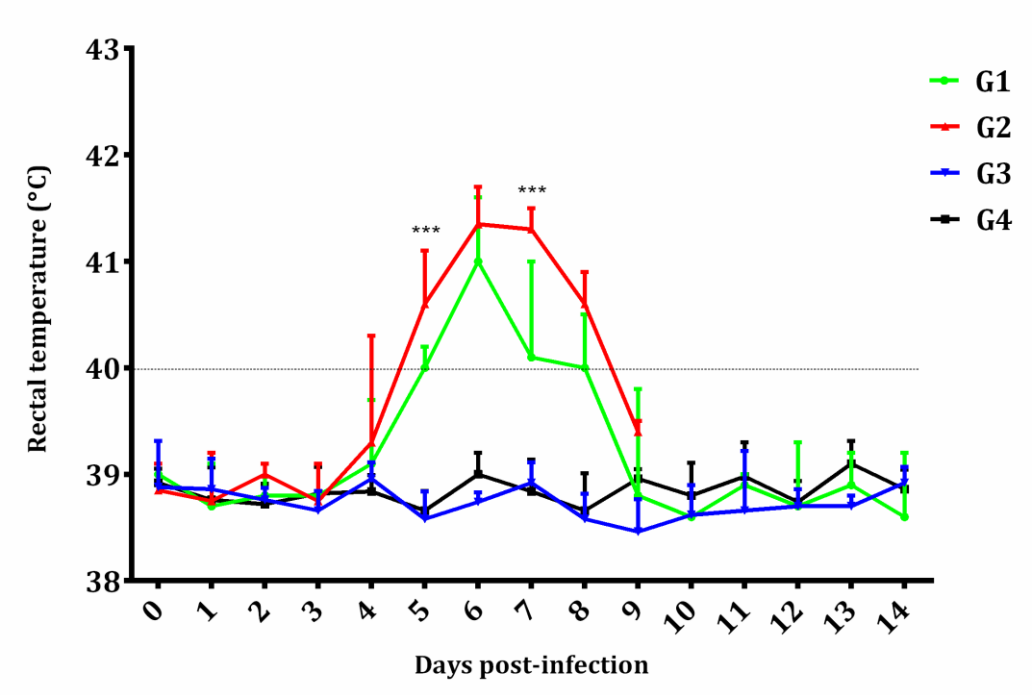
1294 treated group (G3) and ewes gave birth healthy lambs between 146 and 150 days of pregnancy. Dams from the uninfected group but dosed with vehicle alone (G4) gave birth healthy lambs between days 146 and 150 of pregnancy (Figure 3) (Additional file 1).

Concerning the infected groups, statistically significantly increased rectal temperatures were found between days 4 ($P < 0.05$) and 8 pi ($P < 0.001$) in the untreated G2 compared to G4. However, compared to G4, increased rectal temperatures were found in treated G1 between days 5 ($P < 0.0001$) and 8 pi ($P < 0.05$). Comparing both infected groups, a significant decrease was observed in G1 on days 5 ($P < 0.001$) and 7 pi ($P < 0.001$) (Figure 2). Severely reduced voluntary food intake was found in *T. gondii* infected animals from 6 to 11 days pi. From day 14 pi until the end of the experiment, no changes were found in rectal temperatures or voluntary food intake.

Perinatal mortality was detected in 2 out of 7 ewes from infected and BKI-1294 treated group (G1). One ewe aborted three foetuses on day 17 pi and a one ewe gave birth prematurely a stillbirth on day 140 of pregnancy (50 days pi). Therefore, perinatal mortality was found in 4 out 17 foetuses/lambs. However, in infected but untreated group (G2), 8 out of 8 pregnant ewes suffered early abortions (on days 8 and 9 pi), thus, during the acute phase of the disease. Significant differences were found in the perinatal survival curve between G1 and G2 ($P < 0.001$) (Figure 3) and also in the number of lambs born in G1 and G2 ($P < 0.0001$) (Additional file 1). The remaining dams from infected and BKI-1294 treated group (G1) gave birth to 11 healthy and 2 dead lambs due to dystocia (in a quadruplet pregnancy) on days 144 and 150 of pregnancy.

The birthweight of the lambs born from sole pregnancies was 4631.66 ± 347.55 g in G3 and 4504.66 ± 585.27 g in G4. In twin pregnancies, birthweight of the lamb born was 3217 ± 340.61 g in G1, 3867.5 ± 682.35 g in G3 and 3962 ± 487.29 in G4. Finally, in triplet and quadruplet pregnancies, birthweight of the lambs born was 2918.57 ± 398.27 g in G1 and 3228.33 ± 154.94 g in G3. The only significant difference was the lower birthweight of lambs born from twin pregnancies in G1 compared to birthweight of lambs born from twin

Figure 2 - Rectal temperatures of infected (G1 and G2) and uninfected groups (G3 and G4). In infected groups, G1 received BKI-1294 treatment and G2 did not. In uninfected groups, G3 were dosed with BKI-1294 while G4 received vehicle alone. Each point represents the mean + S.D. for each group. Rectal temperatures were analysed using one-way ANOVA followed by Tukey's multiple test. For significant differences, (***) indicates $P < 0.001$.



pregnancies in G4 ($P < 0.05$). However, no significant differences on the birthweight were found in G1 between PCR-positive lambs and PCR-negative lambs in the brain.

3.3. Haematology and biochemistry

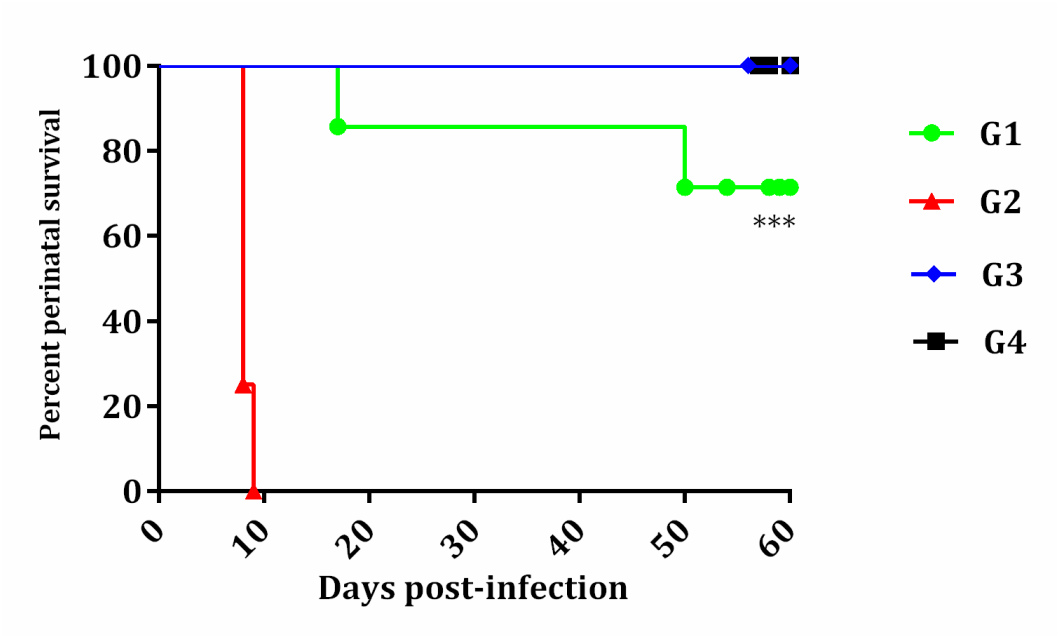
Means and standard deviations for each group and reference values for haematological and biochemical parameters at initial and final time points are shown in Table 2. Mean values for haematological and biochemical parameters were in the physiological range at initial and final time points. The only exception concerned the CK in G4 at final time point, which showed a mean value above the reference.

3.4. Cellular and humoral immune responses

IFNγ levels in supernatants of blood cell cultures recovered 24 hours after *T. gondii* antigen stimulation were significantly increased in samples from G1 isolated on day 7 pi ($P < 0.01$) compared to G2 and G4, with the maximum IFNγ levels on day 10 pi. Furthermore, IFNγ levels in G1 maintained a 100-fold increase compared to G4 at the end of the sampling period. In G2, despite no significant increase was found compared to G4, a 10-fold increase was found on the IFNγ levels on days 5 and 7 pi. In contrast, blood cell cultures from uninfected and untreated animals (G4) showed IFNγ levels throughout the entire experimental study that corresponded to the basal levels recorded prior to inoculation (Figure 4).

The *Toxoplasma*-specific IgG responses in dams are shown in Figure 5. In G1, ewe aborted on day 17 pi were seronegative by both ELISAs. The remaining ewes were seropositive by the SAG1 commercial ELISA (2 dams were seropositive from day 21 pi, 1 dam was

Figure 3 - Kaplan–Meier survival curves for foetuses in the infected groups (G1 and G2) and uninfected groups (G3 and G4). In infected groups, G1 received BKI-1294 treatment and G2 did not. In uninfected groups, G3 were dosed with BKI-1294 while G4 received vehicle alone. Each point represents the percentage of surviving animals at that day, and downward steps correspond with observed deaths. Foetal survival curves were compared by the Log-rank (Mantel-Cox) test. For significant differences between foetal survival curves of infected groups, (***) indicates $P < 0.001$.



seropositive from day 28 pi and the remaining 3 dams in this group were seropositive from day 35 pi) (Figure 5A). However, only the ewe that gave birth a stillborn lamb and 2 ewes that gave birth healthy but congenitally infected lambs were seropositive on days 42-49 pi by ELISA based on *T. gondii* soluble antigen (Figure 5B). All animals from G2 and G4 were seronegative by both ELISAs throughout the experimental study.

Aborted foetuses in G1 and G2, as well as lambs born alive in G1, were all seronegative, except the stillbirth detected in G1 with an IFAT titre of 1:256 (Table 3) (Additional file 1). Specific IgG responses against parasite antigen were not detected in foetuses/lambs from the group receiving vehicle alone (G4).

3.5. Histopathological examination

Multifocal non purulent encephalitis was only found in the brain from the stillborn lamb found in G1. This lesion was characterized by diffuse congestion in the white matter and several glial foci with central area of necrosis randomly distributed in the brain. The brains from foetuses aborted on day 17 pi were too autolytic to allow proper histological evaluation. No significant lesion was found in the brain of the lambs born healthy in G1. Likewise, no significant lesion was found in the lungs from the foetuses aborted, the stillborn lamb and the lambs born healthy in G1.

In G2, the brain from foetuses were too autolytic to allow proper histological evaluation. No significant lesions were found in the lungs from these foetuses. Finally, no histopathological findings were found in the uninfected group (G4).

Table 2 - Haematological and biochemical parameters at initial and final time points.

Parameter (units)	Reference values	G3 (uninfected/treated)		G4 (uninfected/ vehicle alone)	
		Initial	Final	Initial	Final
Erythrocytes (x10^6)	9-14	10.68 ± 0.67	9.26 ± 0.57	10,74 ± 0.55	9,04 ± 0.66
Haemoglobin (g/dL)	8-15	11.78 ± 0.40	10.36 ± 0.78	12.24 ± 1.04	10.38 ± 0.86
Packed cell volume (%)	28-40	32.74 ± 1.96	28.52 ± 1.49	32.94 ± 2.01	27.94 ± 1.89
Platelets (x10^3)	250-750	394.20 ± 97.85	533.80 ± 121.61	583.4 ± 73.67	615 ± 114.58
Leukocytes (x10^3)	4-12	4.85 ± 1.12	4.84 ± 0.63	7,21 ± 1.42	6.53 ± 1.98
Segment neutrophils (%)	10-50	38.72 ± 4.86	41.50 ± 12.58	45.3 ± 5.13	38.24 ± 9.39
Lymphocytes (%)	40-75	51.44 ± 4.04	46.84 ± 13.13	45 ± 5.77	51.48 ± 11
Monocytes (%)	1-6	3.70 ± 1.04	5.28 ± 1.99	4.12 ± 1.14	4.46 ± 1.47
Eosinophils (%)	0-15	3.62 ± 1.47	4.04 ± 2.43	1.88 ± 0.94	2.38 ± 1.73
Proteins (g/dL)	6-8	6.42 ± 0.34	6.56 ± 0.26	6.8 ± 0.36	6.96 ± 0.32
AST (UI/L)	70-210	97.80 ± 27.34	82.60 ± 27.27	97.4 ± 15.24	119 ± 39.05
GGT (UI/L)	36-93	56.60 ± 9.65	61.80 ± 12.07	66 ± 11.95	64 ± 7.71
ALP (UI/L)	44-355	191.40 ± 62.79	221.80 ± 64.61	304.2 ± 60.60	266.4 ± 77.18
CK (UI/L)	50-180	91.75 ± 4.78	143.75 ± 60.11	170.66 ± 47.81	301 ± 151.70
Urea (mg/dL)	8.4-30.8	12.08 ± 1.60	15.36 ± 3.46	15.46 ± 1.16	12.02 ± 4.18
Creatinine (mg/dL)	0.9-1.7	0.98 ± 0.04	0.9 ± 0.04	1 ± 0.07	1.22 ± 0.08
Calcium (mg/dL)	7.1-9.8	9.80 ± 0.68	10.24 ± 0.75	10.22 ± 0.50	9.76 ± 0.50
Phosphorus (mg/dL)	3.5-7.3	4.98 ± 1.03	7.18 ± 0.95	6.06 ± 0.35	5.9 ± 0.38
Sodium (mEq/L)	139-152	147.20 ± 0.83	150 ± 2	148.8 ± 1.30	146 ± 1.22
Potassium (mEq/L)	3.9-5.2	4.64 ± 0.24	4.94 ± 0.33	4.7 ± 0.46	4.92 ± 0.18

3.6. Parasite detection in foetal tissues

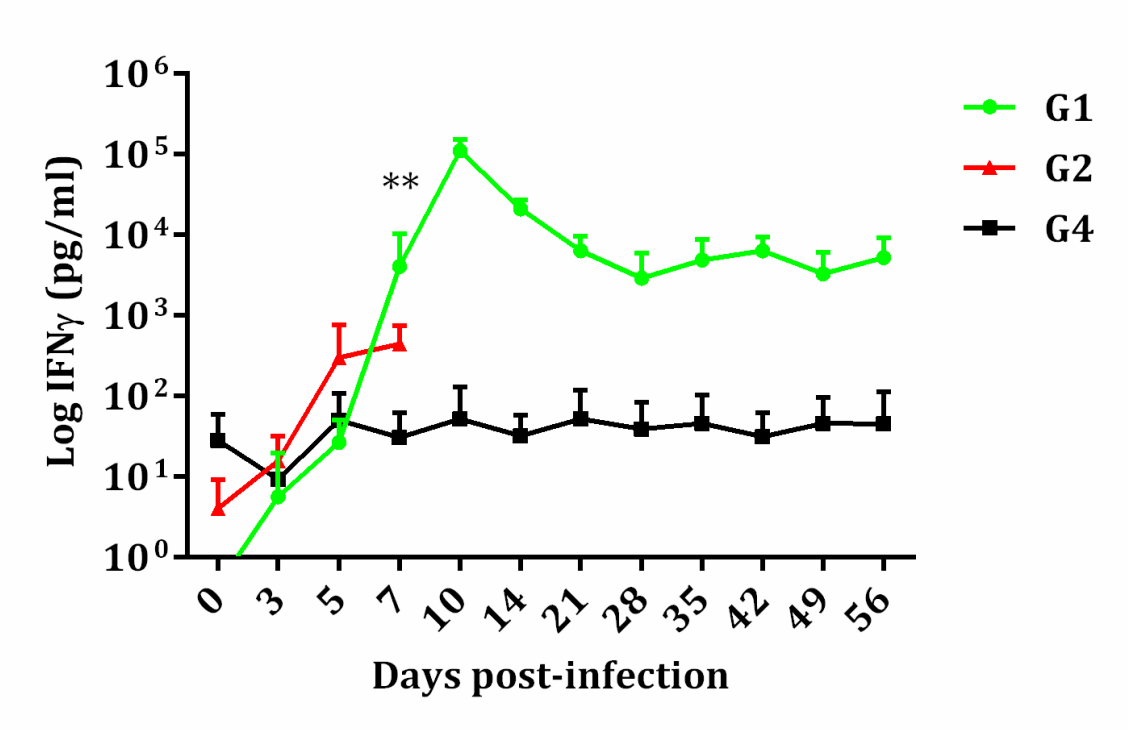
In G1, *T. gondii* DNA was detected in all brain samples from the stillbirth and in 66% of the brain samples from one of the three foetuses aborted on day 17 pi. However, *T. gondii* DNA was not detected in the lung samples from the aborted foetus or the stillbirth. In seven out of thirteen lambs born healthy in G1 *T. gondii* was not detected in the brain or lung samples. Two dams gave birth all the lambs with no detection of *T. gondii* in the brain. However, 33% and 66% of brain positive samples were detected in five and one of the thirteen lambs born healthy, respectively (Table 3) (Additional file 1). In G2, *T. gondii* DNA was not detected in any of the foetal brain samples and only in 2/51 (4%) of foetal lung samples (Additional file 1). As expected, all foetal samples from G4 were negative.

4. Discussion

Previous studies on drugs against *T. gondii* in pregnant sheep showed low efficacy against abortion and/or vertical transmission (Buxton *et al.*, 1987; Buxton *et al.*, 1988; Buxton *et al.*, 1993a; Buxton *et al.*, 1996). Therefore, studies on efficacy of drug target candidates against *T. gondii* are needed (Müller and Hemphill, 2013). This study reports on BKI-1294 drug levels in plasma of pregnant sheep, and the safety and anti-parasitic efficacy of BKI-1294 treatment in a pregnant sheep model of toxoplasmosis. The efficacy was assessed with respect to the clinical course of disease, cellular and humoral immune responses and parasite detection and lesions in foetal tissues.

Compared to other BKIs, such as BKI-1553, BKI-1294 showed lower plasma concentrations in mice (Schaefer *et al.*, 2016; Vidadala *et al.*, 2016).

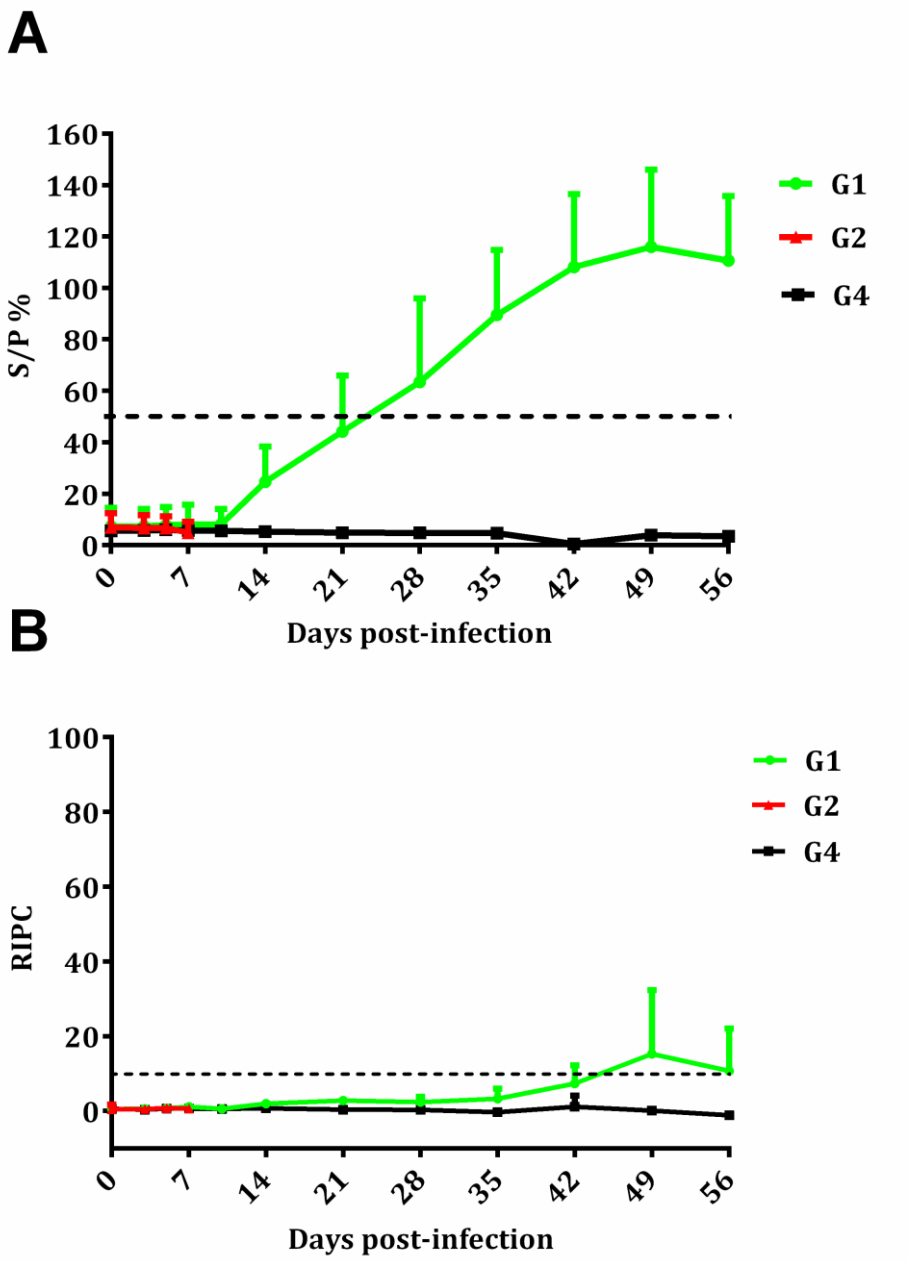
Figure 4 - IFNγ in supernatants of peripheral blood cell cultures. Values from infected groups (G1 and G2) and group receiving vehicle alone (G4). Each point represents the mean + S.D. at the different sampling times for each group. Concentrations of IFNγ are expressed in pg/mL. Cellular immune responses represented in the figure were analysed using two-way ANOVA of repeated measures until day 7 pi. For significant differences between infected groups, (**) indicates $P < 0.01$.



However, BKI-1294 showed levels of 0.75 µM after oral administration of 10 mg/kg in mice (Schaefer *et al.*, 2016; Hulverson *et al.*, 2017). After oral multidoses in mice at 40 mg/kg of BKI-1294 orally, 3 times a day for 4 consecutive days, plasma levels of 2-6.3 µM were found (Ojo *et al.*, 2013). In calves, administration of BKI-1294 at 10 mg/kg resulted in plasma levels of 1 µM until 24 hours after treatment (Schaefer *et al.*, 2016). In this experiment, after administration of 100 mg/kg of BKI-1294 in pregnant sheep, maximum concentrations of 2 µM, with trough plasma concentrations of 0.4-1 µM at 48 hours after treatment were detected. However, after the 4th and 5th doses in infected and treated group (G1), higher plasma concentrations were detected, most likely due to increased absorption derived from the decreased food intake (associated with *T. gondii* fever peak) as previously described Hennessy *et al.* (1995) for treatment of gastrointestinal nematodes in sheep with albendazole. Detectable drug levels were found until 13-17 days after the first dose. Previous studies addressing the *in vitro* efficacy of BKI-1294 against the reference type II *T. gondii* isolate (TgME49) reported a half-maximal *inhibitory concentration* (IC_{50}) of 0.22 ± 0.06 µM (Winzer *et al.*, 2015). Thus, plasma concentrations of BKI-1294 in pregnant ewes were higher than the IC_{50} for *T. gondii*, perhaps indicating adequate exposure that could translate into good efficacy in the pregnant sheep model of toxoplasmosis.

BKI-1294 at 100 mg/kg twice daily for 5 days in mice did not show any signs of toxicity such as weight loss or alterations in tissue histology, metabolic enzymes, and complete blood counts (Ojo *et al.*, 2013). Concerning safety during pregnancy, decrease by half on fertility was found in pregnant CD1 mice treated with BKI-1294 at 50 mg/kg for 5 days (Müller *et al.*, 2017c), however, no detrimental

Figure 5 - IgG responses in sera by ELISA *T. gondii* SAG1 protein (A) or soluble antigen (B). Values from infected groups (G1 and G2) and group receiving vehicle alone (G4). Each point represents the mean + S.D. at the different sampling times for each group.



effect on fertility was found using BALB/c mice (Winzer *et al.*, 2015). In calves, BKI-1553, related to BKI-1294, was safe with no toxicity observed after oral administration (Vidadala *et al.*, 2016). In this study, BKI-1294 did not show increase on rectal temperature nor alterations on the hematological and biochemical parameters. The only exception was an increase on CK values in G4 at final time point, although increased CK values can typically appear in late pregnancy (Yokus *et al.*, 2006). In spite of the decrease by the half on fertility in mice, no abortions were found in pregnant sheep treated with BKI-1294 and all lambs born healthy with no decrease on birthweight. Concerning local toxicity, no alterations on the faecal consistency was found. Therefore, administration in sheep at mid-

pregnancy of 5 oral doses of BKI-1294 at 100 mg/kg (formulated in 60% PHOSAL® 53 MCT, 30% Polyethyleneglycol 400 and 10% Ethanol 96º) seems to be safe.

Previous drugs tested against *T. gondii* in pregnant sheep were dosed starting 10 days before the infection until parturition (Buxton *et al.*, 1987; Buxton *et al.*, 1988; Buxton *et al.*, 1996). However, initiation of BKI-1294 treatment 48 h after infection was scheduled based on experience with BKI therapy of toxoplasmosis in mice (Doggett *et al.*, 2014; Huang *et al.*, 2015; Müller *et al.*, 2017c). Hence, when a robust infection and dissemination of the parasite in the sheep has already taken place. Since type II *T. gondii* isolates are the most frequent in sheep worldwide (Sharif *et al.*, 2017), challenge was carried out with a type II (genotype #3) *T. gondii* isolate, TgShSp1, isolated from an ovine abortion outbreak (Sánchez-Sánchez et al., submitted). Although 500 sporulated oocysts of TgShSp1 isolate triggered 100% abortion (Sánchez-Sánchez et al., submitted), infection with 2000 sporulated oocysts of TgShSp1 resulted in 5 aborted ewes and 1 ewe that gave birth (unpublished data). In consequence, to have a large number of abortions in the untreated ewes, 1000 sporulated oocysts were applied at 90 days of pregnancy. In this experiment, pregnant ewes infected but not treated showed 100% perinatal mortality as previously described Sánchez-Sánchez et al. (submitted). Clinical observations in the BKI-1294 treated group revealed a high protection against perinatal mortality, as 13 out of 17 foetuses/lambs (76%) were born, with statistically improved perinatal survival rates compared to infected but not treated group. Also, a notable delay from day 8-9 pi (acute abortions in infected but untreatred ewes) to days 17-50 pi was found on the time in which perinatal mortality occurred in 2 infected and treated ewes. Although previous drugs tested orally against *T. gondii* in pregnant sheep were dosed starting 10 days before the infection until parturition, allowing better chance to control *T. gondii* infection, in this study, treatment with 5 doses of BKI-1294 initiated 48 hours after infection resulted in higher protection against perinatal mortality (76%) compared to studies using monensin (38% of increase in live lambs) (Buxton *et al.*, 1988) and decoquinate (22% of protection against abortions) (Buxton *et al.*, 1996). Likewise, contrary to this study in which 100% perinatal mortality was found in infected but untreated pregnant ewes, only 53-55% of perinatal mortality was detected in infected but untreated pregnant ewes from studies testing monensin and decoquinate (Buxton *et al.*, 1988; Buxton *et al.*, 1996), indicating less aggressive infection. Protection against perinatal mortality in this study is also higher than this achieved with BKI-1553 (37-50% of protection against abortion) in a pregnant sheep model of neosporosis (Sánchez-Sánchez *et al.*, 2018). In previous studies, a decreased birthweight has been described in lambs born form *T. gondii* infected ewes (Buxton *et al.*, 1996). In this study, lambs born from twin pregnancies in group infected and treated exhibited slightly lower birthweights compared to uninfected group receiving vehicle alone, but since no significant differences were found between lambs PCR-positive and PCR-negative in the brain, is likely that this lower growth of the foetuses could be consequence of a severely decrease on the food intake in the dams from infected and treated group for 6 days (from 96 to 101 days of pregnancy, associated to marked fever peak due to *T. gondii* infection) as previously described Gardner *et al.* (2007). Likewise, all lambs born healthy while in previous studies using TgShSp1 in pregnant sheep, weak lambs were often observed (Sánchez-Sánchez et al., submitted). Rectal temperatures in pregnant ewes infected but non-treated increased from day 4 to day 8 pi, likely as a consequence of tachyzoite multiplication and the first cycles of parasite replication in host tissues, similar to previously reported results in sheep experimentally infected with 500 oocysts of the TgShSp1 isolate (Sánchez-Sánchez et al., submitted).

Table 3 - Parasite detection and serology of foetuses/lambs from ewes infected with *T. gondii* and treated with BKI-1294.

Perinatal viability	Ewes with *T. gondii* positive offspring[a]	Foetuses/lambs		
		IFAT[b]	PCR[c]	
			Brain	Lung
Abortion	1/1	0/3	1/3 (2/9)	0/3 (0/9)
Stillbirths	1/1	1/1 (1:256)	1/1 (3/3)	0/1 (0/3)
Live lambs	3/5	0/13	6/13 (7/39)	0/13 (0/39)

[a] Number of ewes with at least one foetus/lamb positive by serology or PCR/total number of ewes.
[b] Number of foetuses/lambs being *T. gondii* seropositive by IFAT/total number of foetuses or lambs. In brackets, the positive IFAT titres.
[c] Number of foetuses/lambs with at least one positive sample by PCR/total number of foetuses or lambs. In brackets, the parasite detection (PCR-positive samples/total number of samples analyzed)

The rectal temperatures from infected and treated pregnant ewes were lower than infected but untreated pregnant ewes on days 5 and 7 pi, suggesting that the drug had an impact on parasite replication. Likewise, one day of delay on the onset of rectal temperature increase was found in infected and treated ewes compared to those infected but untreated. Decreased rectal temperatures and/or delay in on the onset of rectal temperatures increase have also been described in experiments testing monensin (Buxton *et al.*, 1988) or decoquinate (Buxton *et al.*, 1996) against toxoplasmosis in pregnant ewes.

IFNγ is known to be important in inhibiting the intracellular multiplication of *T. gondii* and in addition will create the appropriate cytokine microenvironment for the priming of the adaptive immune response towards a Th-1 type pro-inflammatory immune response (Innes and Vermeulen, 2006). Analysis of the peripheral immune responses in pregnant ewes at different time points demonstrated a significant increase on the IFNγ release in stimulated peripheral blood cultures from infected and treated group on day 7 pi compared to infected but not treated group and uninfected group receiving vehicle alone. Likewise, infected and treated group showed a peak of IFNγ release on day 10 pi as well as 100-fold increased levels until delivery. *In vitro* studies showed that BKI-1294 induced the formation of intracellular multinucleated complexes composed of multiple pre-zoites unable to separate and form tachyzoites, but remaining viable for extended periods of time. These multinucleated complexes exhibit increased tachyzoite specific antigen1 (SAG1) expression, and also increased expression of the bradyzoite marker BAG1, with an overall heavily distorted parasite ultrastructure (Winzer *et al.*, 2015). If such complexes are also formed *in vivo*, they are unlikely to evade immune responses, but would be increasingly exposed to antigen-presenting cells, which would then result in higher IFNγ levels in treated animals, as previously found (Sánchez-Sánchez *et al.*, 2018) after treatment with BKI-1553 of *N. caninum* experimentally infected pregnant ewes. The SAG1 molecule is an immunodominant surface protein found on tachyzoites and is one of the most extensively studied antigens as it is able of inducing a T cell response with parasiticidal activity for extracellular *T. gondii* tachyzoites (Khan *et al.*, 1988). Therefore, and similarly to the enhanced to potency of drugs against *T. gondii* observed *in vitro* (Radke *et al.*, 2018) and *in vivo* (Araujo and Remington, 1991) in the presence of IFNγ, the effect of BKI-1294 on *T. gondii* could act

synergistically with IFNγ response. The increased levels of IFNγ in infected and treated group might have led to greater initial control of parasitaemia at the peripheral level, diminishing the numbers of parasites reaching and invading the placenta (Entrican, 2002).

Concerning humoral immune responses, all animals infected and treated (G1) were seropositive using SAG1 antigen except one ewe that aborted on day 17 pi. However, by the ELISA based on *T. gondii* soluble antigens only one ewe that gave birth a stillborn lamb and two dams that gave healthy lambs seroconverted, while the remaining ewes were seronegative at the end of the sampling period. Since soluble antigens are exposed to the immune system during replication (Joiner and Roos, 2002), and since much lower humoral immune response to soluble antigens was found compared to ewes infected with lower oocyst doses (Sánchez-Sánchez, et al., submitted), it suggests low replication of the parasite throughout the experiment. Presence of SAG1 antibodies maybe derived from SAG1 expression in multinucleated complexes found *in vitro* after BKI-1294 treatment of *T. gondii* infected cultures (Winzer *et al.*, 2015). Likewise, it is known that the SAG1 antigen triggers an antibody response with an inhibitory effect on invasion (Mineo *et al.*, 1993).

In previous studies testing drugs against congenital toxoplasmosis in pregnant sheep, vertical transmission was evaluated through foetal serology and microscopic observation of lesions in placental tissues and foetal brains, with around 50% less of animals with vertical transmission after treatment with monensin (Buxton *et al.*, 1988), or only by evaluating placental tissues with 50% less of placentas showing lesions using decoquinate (Buxton *et al.*, 1996), albeit around 12% of infected and untreated sheep did not show placental lesions (Buxton *et al.*, 1988; Buxton *et al.*, 1996) or seropositive offspring (Buxton *et al.*, 1988). However, the study evaluating sulphamezathine/pyrimethamine showed 100% vertical transmission in untreated animals and 50% reduction on the placentas with lesions, but not differences on foetal serology in treated ones (Buxton *et al.*, 1993a). In the present study we evaluated transplacental transmission of the parasite through foetal serology and parasite detection and histological lesions in foetal tissues. In all the lambs born after infection with a 100-fold lower TgShSp1 oocyst dose than used in this study, *T. gondii* was detected in all their brains and lungs and lesions was found in most of the brains (Sánchez-Sánchez et al., submitted). However, in lungs from lambs born in BKI-1294 treated group, known as a predilection site for *T. gondii* (Gutierrez *et al.*, 2010), no significant lesions or parasites were detected. Likewise, in foetal brains parasites were not detected in 7 lambs born healthy and slightly detected in the brain of 6 of the 13 lambs born healthy (53% of protection against vertical transmission in lambs born), while no lesions in the brain were found in any of them. Therefore, there was a slight dissemination of the parasite in the lambs with no antibodies detected by IFAT, although this technique could suffer of low sensitivity (Castaño *et al.*, 2016). These results are consistent with those from a pregnant mouse model of toxoplasmosis, in which 100% protection against perinatal mortality and 93% of protection against vertical transmission in the surviving offspring were accomplished with BKI-1294 (Müller *et al.*, 2017c). The higher protection of BKI-1294 in pregnant mice compared to pregnant sheep could be explained by the lower perinatal mortality and vertical transmission of *T. gondii* type II isolates in mice compared to sheep (Sánchez-Sánchez et al., submitted). Since BKI-1294 triggers formation of multinucleated complexes in *T. gondii* infected cultures (Winzer *et al.*, 2015), maybe *in vivo*, is likely that some tachyzoites trapped within the host cell could restart their replication and therefore induce perinatal mortality or vertical transmission. In fact, in some ewes the parasites reached the foetus, but since it is likely that therapeutic concentrations of BKI-1294 might have been

reached in the foetus, similarly to the BKI-1553 (Sánchez-Sánchez *et al.*, 2018), the dissemination of the parasite could be controlled. However, probably the central nervous system penetration of the drug did not allow drug levels above IC_{50} in the foetal brain, which resulted in replication of the parasite in some cases.

In conclusion, BKI-1294 treatment in *T. gondii* infected dams resulted in a decrease on rectal temperatures upon infection, a strong IFNγ production, a low humoral immune response to soluble antigens but high levels of SAG1 antibodies and a decrease of 76% in the perinatal mortality. In the offspring, BKI-1294 prevented vertical transmission in 53% of lambs born. Results of protection against abortion and vertical transmission of BKI-1294 are very similar to those reported experimentally after immunization with the commercial live vaccine (Buxton *et al.*, 1991), and also to those found with the Mic1-3 Knockout of *T. gondii* (Mévélec *et al.*, 2010). In the light of these findings, BKI-1294 exhibits a therapeutic systemic exposure in pregnant ewes, is safe and confers high protection against abortion and vertical transmission of the parasite in a pregnant sheep model of toxoplasmosis. Further studies are necessary to improve efficacy of BKI-1294, by applying alternative formulations and using other routes of administration, drug dosages and dosing regimes. In addition, other members of the BKI class of compounds under development could be tested in the near future against ruminant toxoplasmosis.

Competing interests

The authors declare that they have no competing interests.

Author contributions

IF, AH, KO, WVV and LMO conceived the study and participated in its design. RSS wrote the manuscript, with results interpretation and discussion inputs from IF, JRC, AH, MH, LB, WVV and LMO. LMF selected the animals and executed the reproductive programme. RSS, IF and MR carried out oocyst infection and drug administration. RSS, IF, MR, MPD, MGH, ET and JB participated in clinical examination, sampling of animals and performed necropsies and haematological, biochemical and histopathological analyses. MH, LB, RC, GW, KO and WVV determined the pharmacokinetics of the compound. RSS performed peripheral blood stimulation assay, serological assays, PCR analyses, statistical analysis and interpreted the results. All authors read and approved the final manuscript.

Acknowledgements

We gratefully acknowledge Irene Écija and Juan José de Andres from the SALUVET group (Complutense University of Madrid, Spain), José Calasanz Jiménez, Francisco Saura, Teresa Navarro and José María González (University of Zaragoza, Spain) and Victor Herrero and Javier Blanco from the Clinical Veterinary Hospital (Complutense University of Madrid, Spain) for their excellent technical assistance. The Animal Experimentation Service (SEA) at the University of Zaragoza is acknowledged for providing their facilities to carry out the reproduction programme. Roberto Sánchez Sánchez is supported by a fellowship from the Spanish Ministry of Education, Culture and Sports (MECD), as a part of the Program of Training of University Teaching Staff (FPU, grant number FPU13/03438). This work was supported by the Public Health Service, National Institutes of Health, Bethesda, MD (grants R01 AI 111341 and R01 HD 080670), the U.S. Department of Agriculture (grant 2014-67015-22106) and the Community of Madrid, Spain (PLATESA, S2013/ABI2906).

Additional file 1 - Individual serological titres in foetuses/lambs and detection of parasite DNA in foetal tissues.

Group	Ewe ref.	Foetal death (days p.i)[a]	Foetus/lamb ref.	IFAT[b]	Parasite detection[c]	
					Foetal brain	Foetal lung
Group 1	1.1		1.1 F1	-	-	-
			1.1 F2	-	+	-
			1.1 F3	-	++	-
	1.2		1.2 F1	-	+	-
			1.2 F2	-	+	-
			1.2 F3#	-	-	-
			1.2 F4#	-	-	-
	1.3		1.3 F1	-	-	-
			1.3 F2	-	-	-
	1.4		1.4 F1	-	+	-
			1.4 F2	-	+	-
	1.5	17	1.5 F1	NA	-	-
			1.5 F2	NA	-	-
			1.5 F3	-	++	-
	1.6		1.6 F1	-	-	-
			1.6 F2	-	-	-
	1.7	50 (140 dg)*	1.7 F1	1:256	+++	-
Group 2	2.1	9	2.1F1	-	-	-
			2.1F2	-	-	-
			2.1F3	-	-	-
	2.2	8	2.2 F1	-	-	-
	2.3	8	2.3 F1	-	-	-
			2.3 F2	-	-	-
			2.3 F3	-	-	+
	2.4	9	2.4 F1	-	-	-
			2.4 F2	-	-	-
	2.5	8	2.5 F1	-	-	-
	2.6	8	2.6 F1	-	-	-
			2.6 F2	-	-	+
	2.7	8	2.7 F1	-	-	-
			2.7 F2	-	-	-
			2.7 F3	-	-	-
	2.8	8	2.8 F1	-	-	-
			2.8 F2	-	-	-

[a] Day post-challenge when foetal death was detected by ultrasonography. The remaining foetuses lived until the end of the experiment.

[b] IFAT IgG antibody titres in foetal body fluids and in precolostral serum collected after birth in lambs born alive.

[c] Parasite DNA detection; plus (+++, ++, +) and minus (-) signs represent PCR detection in >67%, 66-34%, <33% and 0% of samples analysed, respectively.

* Premature delivery with a stillborn lamb. Thoracic liquid was assessed by IFAT.

[#] Lambs dead due to dystocia.

NA: not available.

IJP: Drugs and Drug Resistance 8 (2018) 112–124

Contents lists available at ScienceDirect

IJP: Drugs and Drug Resistance

journal homepage: www.elsevier.com/locate/ijpddr

Safety and efficacy of the bumped kinase inhibitor BKI-1553 in pregnant sheep experimentally infected with *Neospora caninum* tachyzoites

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Published in *International Journal for Parasitology: Drugs and Drug Resistance* (2nd March 2018). 8 (2018) 112–124. DOI: 10.1016/j.ijpddr.2018.02.003. Presented as oral communication in the COST Action 1307 meeting "Chemotherapy towards diseases caused by endoparasites": "Targeting metabolism and transport in parasites: drugs and delivery systems" (5th- 6th May 2016, Porto, Portugal) and as poster communication in the 4th International Meeting on Apicomplexa in Farm Animals (*Apicowplexa*), (11th-14th October 2017, Madrid, Spain).

Abstract

Neospora caninum is one of the main causes of abortion in cattle, and recent studies have highlighted its relevance as an abortifacient in small ruminants. Vaccines or drugs for the control of neosporosis are lacking. Bumped kinase inhibitors (BKIs), which are ATP-competitive inhibitors of calcium dependent protein kinase 1 (CDPK1), were shown to be highly efficacious against several apicomplexan parasites *in vitro* and in laboratory animal models. We here present the pharmacokinetics, safety and efficacy of BKI-1553 in pregnant ewes and foetuses using a pregnant sheep model of *N. caninum* infection. BKI-1553 showed exposure in pregnant ewes with trough concentrations of approximately 4 µM, and of 1µM in foetuses. Subcutaneous BKI-1553 administration increased rectal temperatures shortly after treatment, and resulted in dermal nodules triggering a slight monocytosis after repeated doses at short intervals. BKI-1553 treatment decreased fever in infected pregnant ewes already after two applications, resulted in a 37-50% reduction in foetal mortality, and modulated immune responses; IFNγ levels were increased early after infection and IgG levels were reduced subsequently. *N. caninum* was abundantly found in placental tissues; however, parasite detection in foetal brain tissue decreased from 94% in the infected/untreated group to 69-71% in the treated groups. In summary, BKI-1553 confers partial protection against abortion in a ruminant experimental model of *N. caninum* infection during pregnancy. In addition, reduced parasite detection, parasite load and lesions in foetal brains were observed.

Keywords: *Neospora caninum*; sheep; pregnancy; treatment; protein kinase inhibitor; BKI-1553

Highlights:

BKI-1553 showed excellent exposure in pregnant ewes and foetuses.

BKI-1553 confers partial protection against abortion in *N. caninum* infected ewes.

Treatment reduces parasite detection, parasite load and lesions in foetal brains.

1. Introduction

Neospora caninum (Apicomplexa: Eimeriina: Sarcocystidae) is an obligate intracellular parasite, known to be one of the most important infectious causes of abortion in cattle worldwide (Dubey and Schares, 2011; Dubey *et al.*, 2017). Since its discovery, *N. caninum* has been identified in various species of livestock, including cattle, sheep, goats, horses and deer (Dubey *et al.*, 2007). Cattle can become infected by horizontal transmission via the ingestion of oocysts, or by vertical transmission (i.e., transplacentally) as a result of either a primary infection of the dam by oocysts (exogenous transplacental transmission) or recrudescence of a chronic infection (endogenous transplacental transmission) during pregnancy, with different clinical and epidemiological consequences (Williams *et al.*, 2009).

The clinical and economic importance of neosporosis in small ruminants has historically been considered much less relevant compared to infection by *Toxoplasma gondii*, which is one of the most common causative agents of abortion in sheep and goats (Dubey, 2009b). However, recent evidence suggests that *N. caninum* is also an important abortifacient in small ruminants (Moreno *et al.*, 2012) and may even be the main cause of reproductive losses

in some flocks (West *et al.*, 2006; González-Warleta *et al.*, 2014). Experimental infections in pregnant sheep (McAllister *et al.*, 1996b; Buxton *et al.*, 1998; Weston *et al.*, 2009; Arranz-Solis *et al.*, 2015b) have shown that they are highly susceptible, and as in cattle, abortion and vertical transmission are the main consequences of infection.

Many control measures have been proposed to reduce *N. caninum* infection in cattle, including embryo transfer, artificial insemination of seropositive dams, culling of infected animals and replacement by healthy heifers, drug treatment and vaccination (Dubey *et al.*, 2007). The latter two options have been identified as economically viable, provided suitable targets and efficacious drugs can be made available (Häsler *et al.*, 2006a; Häsler *et al.*, 2006b). Although experimental studies have revealed potent effects of several drugs *in vitro* and in laboratory animal models (Müller and Hemphill, 2011; Hemphill *et al.*, 2016), only triazinon derivatives, such as ponazuril (Kritzner *et al.*, 2002) and toltrazuril (Haerdi *et al.*, 2006; Syed-Hussain *et al.*, 2015a), and the polyether ionophore antibiotic monensin (Vanleeuwen *et al.*, 2011) have been tested in ruminants experimentally infected with *N. caninum*, but results remained ambiguous. To date, pregnant ruminant models of neosporosis have not been used for assessments of drug efficacy against *N. caninum* infection and vertical transmission.

Anti-parasitic drug development based on targeting kinase enzymes is a well-established approach (Rotella, 2012). Calcium dependent protein kinase 1 (CDPK1) represents a promising drug target, as CDPK1 is encoded by the apicoplast DNA, and is thus absent from mammalian hosts (Lourido *et al.*, 2010; Murphy *et al.*, 2010; Ojo *et al.*, 2010). CDPK1 activity is essential for microneme secretion, host cell invasion, and egress of *T. gondii* (Kieschnick *et al.*, 2001; Lourido *et al.*, 2010) and can be effectively targeted by a class of ATP-competitive compounds, collectively named bumped kinase inhibitors (BKIs).

BKIs have a broad-spectrum activity that affects many apicomplexan parasites (Van Voorhis *et al.*, 2017). BKI-1294, BKI-1517 and BKI-1553 were all effective against *N. caninum in vitro* and strongly interfered with transplacental transmission in a pregnant mouse model of neosporosis (Ojo *et al.*, 2014; Winzer *et al.*, 2015; Müller *et al.*, 2017b). BKI-1553 has been developed based on a variant on the naphthalinyl-pyrazolopyrimidine scaffold of BKI-1294. BKI-1553 is highly efficacious against *T. gondii in vitro*. It exhibits a low human ether-a-go-go-related gene (hERG) ion channel inhibition, excellent systemic exposure, crosses the blood-brain barrier in mice when administered orally, and BKI-1553 treatment lead to reduced parasite burden in the brain, lungs and liver of *T. gondii* infected mice (Vidadala *et al.*, 2016). We here report on the safety and efficacy of BKI-1553 treatment in pregnant sheep experimentally infected with *N. caninum* tachyzoites at mid-gestation, drug levels in foetuses, and its impact on vertical transmission.

2. Materials and methods

2.1. Ethics statement

All protocols involving animals were approved by the Animal Welfare Committee of the Community of Madrid, Spain, following proceedings described in Spanish and EU legislation (PROEX 166/14 -experiment 1- and PROEX 064/15 -experiment 2-, Law 32/2007, R.D. 53/2013, and Council Directive 2010/63/EU). All animals used in this study were handled in strict accordance with good clinical practices, and all efforts were made to minimize suffering.

2.2. Experiment 1: pharmacokinetics, safety and efficacy of BKI-1553 in a pregnant sheep model of neosporosis

2.2.1. Animals and experimental design

Fifty-four pure Rasa Aragonesa breed female lambs aged 3 months were selected from a commercial flock. All animals were seronegative for *T. gondii*, *N. caninum*, Border disease virus (BDV), Schmallenberg virus (SBV), *Coxiella burnetii* and *Chlamydia abortus* as determined by enzyme linked immunosorbent assay (ELISA). Animals were maintained in isolation in Zaragoza University (Spain) facilities until 12 months of age. They were oestrus-synchronized and mated with pure-breed Rasa Aragonesa tups for 2 days, after which the rams were removed from the ewes. Pregnancy and foetal viability were confirmed by ultrasound scanning (US) on day 40 post-mating, and thirty-seven pregnant sheep were selected for the experiment. Pregnant ewes (n = 37) were randomly distributed into six experimental groups (see Table 1) and housed at the Clinical Veterinary Hospital facilities (Complutense University of Madrid, Spain). Twenty-four ewes were allocated into groups 1 (G1; n=8), 3 (G3; n=8) and 5 (G5; n=8), which were inoculated intravenously with 10^6 tachyzoites of the bovine isolate Nc-Spain7 (Regidor-Cerrillo *et al.*, 2008) at day 90 of gestation (dg). The thirteen remaining pregnant ewes were allocated to groups 2 (G2; n=5), 4 (G4; n=5) and 6 (G6; n=3), which received an intravenous inoculum of phosphate-buffered saline (PBS) at 90 dg.

BKI-1553 was synthesized by Sundia Inc. (Shijiazhuang, China) and further purified in the Department of Chemistry of the University of Washington. The drug formulation was prepared by dissolving the compound in 70% Tween 80 (Sigma-Aldrich, Madrid, Spain) and 30% Ethanol 96º (Panreac, Barcelona, Spain) by heating at 60°C and shaking for 3 hours at a final concentration of 69 mg/ml. Starting at 48 hours post-infection, BKI-1553 was administered subcutaneously to G1 (1st dose: 35 mg/kg bodyweight, 2nd dose: 10 mg/kg bodyweight a week later) and G3 (10 mg/kg bodyweight, 7 doses every other day). G2 and G4, which represented the corresponding non-infected treatment controls, received the same doses as G1 and G3, respectively. Ewes from G1 and G2 groups were dosed in their armpits with 13.12 ± 0.70 ml for the 1st dose and 8.37 ± 0.93 ml for the 2nd dose. Ewes from groups G3 and G4 were dosed in their armpits and inguinal regions with 8.37 ± 0.20 ml per dose. Ewes from the non-infected groups G2 and G4 were culled around the time when abortion occurred in the respective *Neospora*-infected groups G1 and G3, providing a negative control for further analyses (see below). Ewes from G6 (non-infected, no drug) were kept alive until the end of the experiment.

2.2.2. Parasites

Tachyzoites of the Nc-Spain7 isolate were routinely maintained in cultured MARC-145 cells as described previously (Regidor-Cerrillo *et al.*, 2010). For the *in vivo* challenge, tachyzoites (passage 14) were recovered from culture flasks when they were still largely intracellular (>80% of undisrupted parasitophorous vacuoles), and infected cells were repeatedly passed through a 25-gauge needle at 4°C. Tachyzoite numbers were determined by Trypan blue exclusion followed by counting in a Neubauer chamber, and parasites were resuspended in PBS at the required dose of 10^6 tachyzoites in a final volume of 1 mL. Infection of ewes was carried out within 30 min of harvesting the parasites from cell culture.

Table 1 - Experimental design

Group	Number of pregnant ewes	Number of foetuses/lambs	Inoculum (i.v.)	Treatment (s.c.)
G1	8	14	Nc-Spain7 10^6 tachyzoites	BKI-1553, 1st dose: 35 mg/kg bodyweight; a week later, a 2nd dose at 10 mg/kg bodyweight
G2	5	7	PBS	BKI-1553, 1st dose: 35 mg/kg bodyweight; a week later, a 2nd dose at 10 mg/kg bodyweight
G3	8	13	Nc-Spain7 10^6 tachyzoites	BKI-1553, 7 doses at 10 mg/kg bodyweight every other day
G4	5	9	PBS	BKI-1553, 7 doses at 10 mg/kg bodyweight every other day
G5	8	13	Nc-Spain7 10^6 tachyzoites	None
G6	3	5	PBS	None

i.v.: intravenous route
s.c.: subcutaneous route

2.2.3. Clinical monitoring

Pregnant ewes were observed daily throughout the entire experimental period. Foetal viability was assessed by US monitoring of foetal heartbeat and movements once a week during the first 14 days post-infection (pi) and then twice weekly until detection of foetal death. Rectal temperatures were recorded daily from day 0 until 14 days pi and then weekly. The physiological range for rectal temperatures in sheep was obtained from Antón and Mayayo (2007), and rectal temperatures above 40°C were considered hyperthermic. Skin lesions after subcutaneous BKI-1553 administration were recorded daily until their resolution.

When foetal death occurred, or immediately after parturition, dams and lambs were first sedated with xylazine (Rompun, Bayer, Mannhein, Germany) and then euthanized by an intravenous overdose of embutramide and mebezonium iodide (T61, Intervet, Salamanca, Spain). In G2 and G4, at least one ewe was culled for each of the three ewes that aborted in G1 and G3 as close as possible to the average day of abortion. Animals from G6 were examined by US every two weeks. Lambs were clinically inspected and weighed immediately after birth and then euthanized.

2.2.4. Collection of blood samples

Blood samples to evaluate peripheral immune responses were collected prior to infection and then weekly by jugular venipuncture into 5 mL vacutainer tubes (Becton Dickinson and Company, Plymouth, UK) with and without lithium heparin as anticoagulant. In addition, haematological and biochemical parameters before (day 0 pi) and after treatment (13 days pi for G1, G2, G5 and G6, and 18 days pi for G3 and G4) were assessed in blood samples collected into 10 mL vacutainer tubes (Becton Dickinson and Company, Plymouth, UK), with ethylenediaminetetraacetic acid (EDTA) as anticoagulant and into 5 mL vacutainer tubes (Becton Dickinson and Company, Plymouth,

UK) without anticoagulant. Tubes without anticoagulant were allowed to clot and were centrifuged to obtain serum samples that were stored at −80°C until analysis.

To determine BKI-1553 exposure, blood samples from the treated groups G1-G4 were collected at multiple time points by jugular venipuncture into 2 mL tubes (Aquisel, Barcelona, Spain) containing lithium heparin. From G1 and G2, blood was collected prior to BKI-1553 administration, after the 35 mg/kg bodyweight dose at 12 hours, 48 hours and 7 days, and after the 10 mg/kg bodyweight dose at 12 and 48 hours and 4, 5, 6, 7, 10, 14 and 21 days. From G3 and G4, blood samples were collected prior to BKI-1553 administration, 12 and 48 hours after the first and second doses, 48 hours after the fourth and sixth doses and finally, 12 and 48 hours and 4, 5, 6, 7, 10, 14 and 21 days after the seventh dose. Heparinised blood samples were centrifuged at 805 x *g* for 30 min at 4°C, and plasma samples were stored at -20°C until analysis by liquid chromatography tandem mass spectrometry (LCMS/MS).

Precolostral serum was collected from lambs and maintained at −80°C for subsequent serological analysis. To prevent any transmission of colostral antibodies from dams, lambs were separated from their mothers immediately after birth, sampled for blood and euthanized.

2.2.5. *Post-mortem* collection of tissue and body fluid samples

Six randomly selected placentomes or cotyledons from aborted/euthanized dams and dams that gave birth, respectively, were recovered from each placenta, transversally cut into 2–3 mm-thick slices, and fixed in 10% formalin for histopathological examination, whereas remaining tissues from these placentomes/cotyledons were stored at −80°C for further DNA extraction. Foetal brains were stored at −80°C for DNA extraction and fixed in 10% formalin for histopathological examination. Foetal thoracic and abdominal fluids were also collected from foetuses and maintained at −80°C for serology.

2.2.6. BKI-1553 pharmacokinetics

BKI-1553 was extracted from the plasma samples using acetonitrile/0.1% formic acid with an internal standard. A standard curve was prepared for comparison and quantification. BKI-1553 was quantified by analysis on a 6460 series triple quadrupole LC-MS/MS (Agilent, Santa Clara, CA). For both doses in groups G1 and G2, and for the first and seventh doses in groups G3 and G4, phamacokinetic (PK), calculations of maximum concentration (C_{max}), and area-under-the-curve (AUC) were determined using Pharsight Phoenix WinNonlin software (Certara, St. Louis, MO).

2.2.7. Haematological and biochemical analyses

Complete blood counts (CBCs), including erythrocytes, haemoglobin, packed cell volume (PCV), platelets, leukocytes, segmented neutrophils, lymphocytes, monocytes and eosinophils, were determined in whole blood using the automated laser-based haematology analyser Advia 120 (Siemens, Healthcare Diagnostics GmbH, Eschborn, Germany). Concerning biochemical parameters, proteins, aspartate aminotransferase (AST), gamma-glutamyltransferase (GGT), alkaline phosphatase (ALP), creatine kinase (CK), urea and creatinine levels were measured in serum using the sequential automatic autoanalyzer Konelab 30 (Thermo Fisher Scientific, Waltham, USA). Ions such as calcium, phosphorus, sodium and potassium were assessed in serum using a Microlyte 3 (Beckman Coulter, Brea, USA). Reference values were obtained from Antón and Mayayo (2007).

2.2.8. Peripheral blood cell stimulation assay and assessment of interferon-gamma (IFNγ) production

To ensure that blood cells retained the capacity to respond to stimulation and to secrete IFNγ, heparinized blood samples were processed within 2 h of collection by mixing 500 µL blood with 500 µL RPMI 1640 medium (Gibco, Paisley, UK) supplemented with 10% foetal bovine serum (FBS; Thermo Fisher Scientific, Waltham, USA) and 100X antibiotic/antimycotic solution (Lonza, Belgium). Blood cells were cultured in 24-well flat-bottom plates (Thermo Fisher Scientific, Waltham, USA) in the presence of either soluble *N. caninum* antigens or concanavalin A (ConA, Sigma-Aldrich, Madrid, Spain), both at final concentrations of 5 µg/mL. All experiments were performed in duplicate. Plates were incubated in a 5% CO_2/37°C/100% humidity atmosphere for 24 h. They were then centrifuged at 1000 x *g* for 10 min at 4°C, and cell-free culture supernatants were stored at −80°C for IFNγ analyses. IFNγ was detected using a commercial bovine enzyme immunoassay kit with a capture monoclonal antibody (MT17.1) showing cross-reactivity with ovine IFNγ (Mabtech AB, Sweden) as previously described Arranz-Solís *et al.* (2016). Mean optical density (OD) for each experimental animal was calculated as the mean OD obtained from each supernatant from the *N. caninum* antigen-stimulated cells divided by the mean OD of the same cells incubated with medium alone (negative control). Afterwards, the mean OD was calculated for each experimental group. Supernatant from ConA-stimulated cells was processed in a similar way as a positive control for stimulation but was not included in the subsequent analysis.

2.2.9. Serological analyses: ELISA and IFAT

Neospora caninum-specific IgG antibody levels were measured using an in-house indirect ELISA. Soluble *N. caninum* antigen was prepared according to (Alvarez-Garcia *et al.*, 2003). 96-well microtiter plates (Thermo Fisher Scientific, Waltham, USA) were coated with 100 µL soluble *N. caninum* antigen (1 µg/mL in 100 mM carbonate buffer pH 9.6) overnight at 4°C. Subsequently, nonspecific binding was blocked by adding 300 µL of 3% bovine serum albumin diluted in PBS (pH 7.4) containing 0.05% Tween 20 (PBS-T). After 2 h incubation at room temperature (RT), plates were washed three times with PBS-T. Serum samples were diluted 1:100 in blocking solution, and 100 µL of this dilution was added to each well and incubated during 1 h at 37°C. In each plate, samples of the same positive and negative control sera were included.

After three washes in PBS-T, 100 µL of horseradish peroxidase-conjugated protein G (Sigma-Aldrich, Madrid, Spain) diluted 1:2000 in PBS-T was added and incubated for 1 h at 37°C. Plates were washed as above before the addition of 100 µL per well of ABTS substrate (Roche, Basilea, Switzerland). The reaction was stopped after 14 min at RT by the addition of 100 µL of 0.3 M oxalic acid, and the optical density (OD) was read at 405 nm (OD405). For each plate, values of the OD were converted into a relative index percent (RIPC) using the following formula: RIPC = (OD405 sample – OD405 negative control)/(OD405 positive control–OD405 negative control) × 100. A RIPC value ≥10 indicates a positive result.

Indirect fluorescent antibody test (IFAT) was used to detect specific IgG anti-*Neospora* antibodies in foetal fluids and precolostral sera as previously described Alvarez-Garcia *et al.* (2003). Foetal fluids and precolostral sera were diluted at two-fold serial dilutions in PBS starting at 1:8 (for foetal fluids) and 1:50 (for precalostral sera) up to the endpoint titre.

Continuous tachyzoite membrane fluorescence at a titre ≥ 8 for foetal fluids or ≥ 50 for precolostral sera was considered a positive reaction.

2.2.10. Histopathology and lesion scoring

After fixation in formalin for five days, tissue samples were processed for histological evaluation. Foetal brain samples were subjected to measurement of histological lesion characteristics through software-assisted analysis of digital pictures as previously described Arranz-Solis *et al.* (2015b). Briefly, the number (foci/cm^2) and average size of lesion foci (ASF), as well as the total area of the lesion (%LES), were calculated.

2.2.11. DNA extraction and PCR for parasite detection and quantification in tissues

Genomic DNA was extracted from 50–100 mg of maternal and foetal tissue samples using the commercial Maxwell® 16 Mouse Tail DNA Purification Kit, developed for the automated Maxwell® 16 System (Promega, Wisconsin, USA), following the manufacturer's recommendations. The concentration of DNA for all samples was determined by spectrophotometry and adjusted to 50–100 ng/μL.

Parasite DNA detection was carried out by a nested-PCR adapted to a single tube from the internal transcribed spacer (ITS1) region of *N. caninum*, using the external primers TgNN1-TgNN2 and internal primers NP1–NP2 as previously described Buxton (1998) and Regidor-Cerrillo *et al.* (2014). Each reaction was performed in a final volume of 25 μL with 5 μL of sample DNA.

PCR analysis was performed for six samples of the placentomes in aborted dams or cotyledons in dams that gave birth and three samples of foetal brain tissues. Moreover, both reactions without a template and DNA samples from the uninfected groups (G2, G4 and G6) were included in each round of DNA extraction and PCR as negative controls. Positive PCR controls with *N. caninum* genomic DNA equivalent to 10, 1 and 0.1 tachyzoites in 100 ng of sheep DNA were also included in each batch of amplifications. Ten μL aliquots of the PCR products were visualized under UV light in 1.5% agarose/ethidium bromide gel to detect the *N. caninum*-specific 247 bp amplification product.

Placenta and foetal brain samples that had tested positive by nested-PCR were adjusted to 20 ng DNA/μL and the parasite load was quantified using real-time PCR. Primer pairs from the *N. caninum* Nc-5 sequence (Collantes-Fernández *et al.*, 2002) were used for parasite quantification, and primers from the β-actin gene (Gutierrez *et al.*, 2012) were used for the quantification of host DNA. Amplification reactions were performed as described by Collantes-Fernández *et al.* (2002) with slight modifications in a final volume of 20 μL using Go Taq® qPCR Master Mix (Promega, Wisconsin, USA), 20 pmol of each primer and 100 ng of DNA in an ABI 7300 Real Time PCR System (Applied Biosystems, California, USA). The *N. caninum* tachyzoite numbers were calculated by interpolating the average Ct values on two standard curves: 1) one curve equivalent to 10^5 to 10^{-1} tachyzoites with 10-fold serial dilutions in a solution of ovine genomic DNA, and 2) a curve of 320, 160, 80, 40, 20, 10, and 5 ng of genomic DNA for ovine DNA quantification. Parasite numbers in tissue samples (parasite burden) were expressed as parasite number/mg ovine tissue. Standard curves for *N. caninum* and sheep DNA showed an average slope of −3.45 and −3.33, respectively, and a $R^2 > 0.99$.

2.3. Experiment 2: foetal pharmacokinetics of BKI-1553

2.3.1. Animals and experimental design

Seven pure Churra breed sheep aged 12 months were selected, oestrus-synchronized and mated as described for Experiment 1. Pregnancy and foetal viability were confirmed by US on day 40 post-mating, and three pregnant sheep at 125 ± 9 days of gestation were selected for the experiment.

2.3.2. Foetal catheterization and clinical monitoring

Pregnant ewes were pre-medicated with flunixin meglumine (Fluvex, SP Veterinaria, Spain), benzylpencillin (Penilevel, ERN Laboratorios, Barcelona, Spain) and gentamicin (Gentamicin 60%, Braun, Barcelona, Spain) following the manufacturer´s recommendations. After 24 hours of fasting, pregnant ewes were induced for general anaesthesia, maintained under inhalation anaesthesia with isofluorane (Isovet, Braun, Barcelona, Spain) and monitored for physiological parameters. After hysterotomy and location of the hind limb of one of the foetuses (Herrera *et al.*, 2012), foetal catheterization of the saphenous vein was performed with the catheter Prowler® Select® Plus, 2,8 F/150 cm (Cordis, California, USA). The catheter was fixed to the abdominal skin of the pregnant ewes. Pregnant ewes were observed, and foetal viability was assessed by US monitoring foetal heartbeat and movements, on a daily basis. At the end of the experiment, pregnant ewes were sedated and euthanized as described above (subsection 2.2.3).

2.3.3. Drug administration

Upon recovery from anaesthesia, BKI-1553 formulated as described in subsection 2.2.1 was administered to pregnant sheep subcutaneously into the right armpit at 10 mg/kg bodyweight.

2.3.4. Collection of samples

Pregnant ewes and one of their foetuses were sampled by jugular venipuncture into 2 mL tubes (Aquisel, Barcelona, Spain) containing lithium heparin as an anticoagulant. Samples were collected prior to BKI-1553 administration, 1, 2, 4, and 8 hours after administration and, if possible, 24 and 30 hours after administration. Heparinised blood samples were processed as described in subsection 2.2.6 to determine BKI-1553 exposure.

2.3.5. Statistical analysis

Occurrence of foetal death was analysed by the Kaplan–Meier survival method. Foetal survival curves were then compared by the Log-rank (Mantel-Cox) test, and the median foetal survival time, i.e., the day at which 50% of the foetuses aborted, was calculated. Areas of the skin lesions, weights of the lambs and antibody responses in foetuses and lambs were compared using the non-parametric Kruskal–Wallis test followed by Dunn's test for comparisons between groups, as well as the Mann–Whitney test for pairwise comparisons. Rectal temperatures were analysed using Two-way ANOVA of repeated measures testing until 14 days pi and One-way ANOVA test afterwards. Haematological and biochemical parameters were compared between groups using One-way ANOVA testing at each time point. Humoral and cellular immune responses for each experimental group were analysed using Two-way ANOVA of repeated measures testing until 21 days pi and One-way ANOVA test afterwards. Differences in C_{max} and AUC for infected versus non-infected and aborted versus non-aborted ewes within the different treatments were evaluated using the Mann–Whitney test for pairwise comparisons.
Differences in frequency of PCR detection of parasite DNA were evaluated using the $\chi 2$ or Fisher's exact F-test. Differences in parasite burdens and histological measurements of lesions were analysed using the non-parametric Kruskal–Wallis test followed by Dunn's test

for comparisons between groups, as well as the Mann–Whitney test for pairwise comparisons. Statistical significance for all analyses was established at $P < 0.05$. All statistical analyses were performed using GraphPad Prism 6.01 software (San Diego, CA, USA).

3. Results

3.1. Experiment 1: plasma concentrations, safety and efficacy of BKI-1553 in a pregnant sheep model of neosporosis

3.1.1. Pharmacokinetics

C_{max} for groups treated weekly (G1 and G2) reached 11.7 ± 5.2 µM at 12 hours after the first dose administration (35 mg/kg) and 9.0 ± 3.7 µM at 12 hours after the second dose (10 mg/kg), with trough plasma concentrations of 4.2 ± 2.9 µM after the first dose and 1.5 ± 1.2 µM at the end of the sampling period, 21 days after the final dose. C_{max} for groups treated at 10 mg/kg every 48 hours (G3 and G4) reached 5.7 ± 2.8 µM at 12 hours after the first dose and 7.7 ± 4.7 µM at 12 hours after the final dose, with trough plasma concentrations of 3.6 ± 2.4 µM after the first dose and 2.2 ± 2.1 µM at the end of the sampling period, 21 days after the final dose.

No significant differences were observed between the infected and uninfected animals in groups receiving equivalent treatments for C_{max} or AUCs. For infected ewes receiving weekly treatment (G1), there was no significant difference between the aborted and not-aborted ewes for the AUCs or the C_{max} for the first or second dose. In infected ewes receiving treatment every 48 hours (G3), there was no significant difference between the aborted and not-aborted ewes for AUC or C_{max} of the first or final dose.

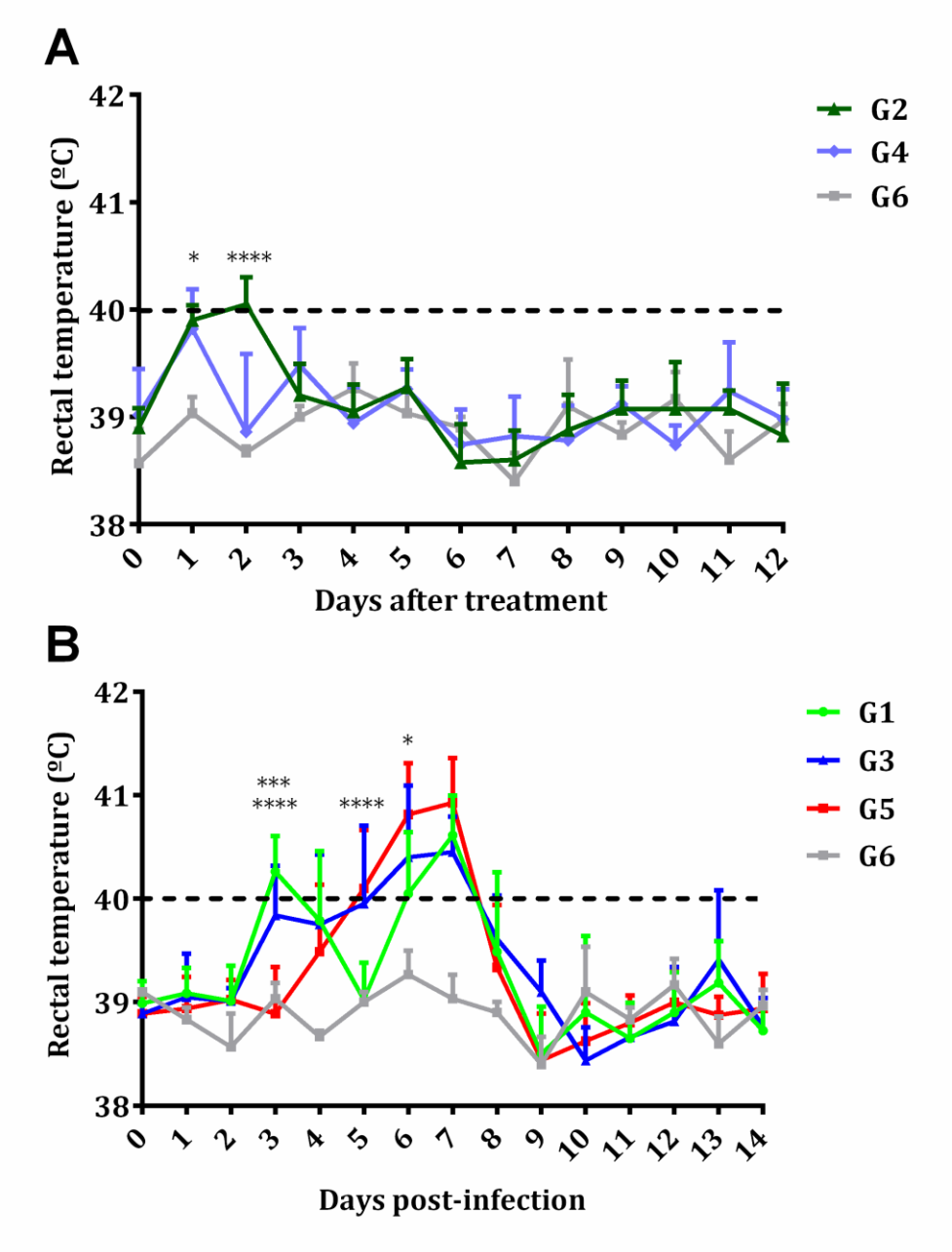
3.1.2. Clinical observations

In sheep that remained uninfected but received treatment (G2 and G4), when analysing the recorded rectal temperatures in relation to G6, a significant increase in G2 was found on day 1 ($P < 0.05$) and day 2 ($P < 0.0001$) after treatment, and in G4 on day 1 after treatment ($P < 0.05$). Mean rectal temperatures in G2 on day 2 were slightly above 40°C (Figure 1A). Foetuses from G2 and G4 remained alive just prior to the euthanasia of two dams on days 41 and 47 pi in G2 and of a dam on day 41 pi in G4. The remaining dams gave birth to 5 healthy lambs in G2 and 7 healthy lambs and 1 dead lamb due to dystocia in G4 between days 145 and 147 of pregnancy.

Concerning the infected groups, statistically significantly increased rectal temperatures were found between days 4 ($P < 0.05$) and 7 pi ($P < 0.0001$) in the untreated G5 compared to G6. Furthermore, compared to G5, rectal temperatures were significantly increased in G1 ($P < 0.0001$) and G3 ($P < 0.001$) on day 3 pi (day 1 after treatment), and a significant decrease was observed in G1 on days 5 ($P < 0.0001$) and 6 pi ($P < 0.05$). Maximum mean rectal temperatures were measured on day 7 pi in all infected groups (Figure 1B). No significant differences in rectal temperatures were found between aborting and non-aborting ewes from G1 and G3. From day 14 pi until the end of the experiment, no changes were found in rectal temperatures.

Dermal nodules at the sites of drug administration were observed 24 hours after application of BKI-1553 in all dams from the

Figure 1 - Rectal temperatures of uninfected groups G2, G4 and G6 (A) and infected groups G1, G3 and G5 and the uninfected/untreated group G6 (B). Each point represents the mean + S.D. for each group. Rectal temperatures represented in the figure were analysed using Two-way ANOVA of repeated measures. For significant differences, (*) indicates $P < 0.05$, (***) indicates $P < 0.001$ and (****) indicates $P < 0.0001$.



treated groups, with areas of 108.8 ± 57.5 cm^2 in G1, 102.1 ± 76.1 cm^2 in G2, 99.6 ± 67.3 cm^2 in G3 and 140.1 ± 66.9 cm^2 in G4, without significant differences between them. These nodules eventually resolved over the course of the experiment.

Foetal death was detected by US between 23-46 days pi in 5 out of 8 pregnant ewes in G1 (median day 39 pi), 4 out of 8 pregnant ewes in G3 (also median day 39 pi) and in all pregnant ewes in G5 (median day 36 pi). Median foetal survival times were 42, 51 and 36 days for G1, G3 and G5, respectively. Significant

differences were found in the foetal survival rate between G3 and G5 ($P < 0.05$). No foetal death was detected in uninfected groups. Dams from the pregnancy control group (G6) gave birth healthy lambs between days 146 and 150 of pregnancy, and the foetal survival rate in G6 was significantly different from G5 ($P < 0.01$) (Figure 2). The remaining dams from G1 gave birth to 6 healthy and 1 dead lamb on days 145 and 146 of pregnancy, whereas the remaining dams in G3 gave birth to 7 healthy and 2 dead lambs on days 142 (premature), 144 and 145 of pregnancy.

Albeit lower, the birthweight of the lambs from G1 (2821.6 ± 360.3 g), G2 (3323 ± 720.6 g) and G4 (2885.8 ± 566.8 g) did not show statistically significant differences compared to G6 (4037.4 ± 354.7 g), while a significant decrease in the birthweight in G3 (1845 ± 434.3 g) was found when compared to G6 ($P < 0.01$) or to G4 ($P < 0.05$).

3.1.3. Haematology and biochemistry

Means and standard deviations for each group and reference values for haematological and biochemical parameters at initial and final time points are shown in Table 2. Mean values for haematological parameters were in the physiological range or showed no significant differences when values from the different groups at initial and final time points were compared. The only exceptions concern the lymphocyte percentage, which showed a significant increase ($P < 0.01$) at the final time point in G5 compared to G3, and the monocyte percentage, which showed a significant increase ($P < 0.05$) at the final time point in G3 compared to G1.

Figure 2 - Kaplan–Meier survival curves for foetuses in the infected groups G1 and G3 treated with BKI-1553, infected group G5, and the non-infected groups. Each point represents the percentage of surviving animals at that day, and downward steps correspond with observed deaths. Foetal survival curves were compared by the Log-rank (Mantel-Cox) test. For significant differences between foetal survival curves of infected groups, (*) indicates $P < 0.05$.

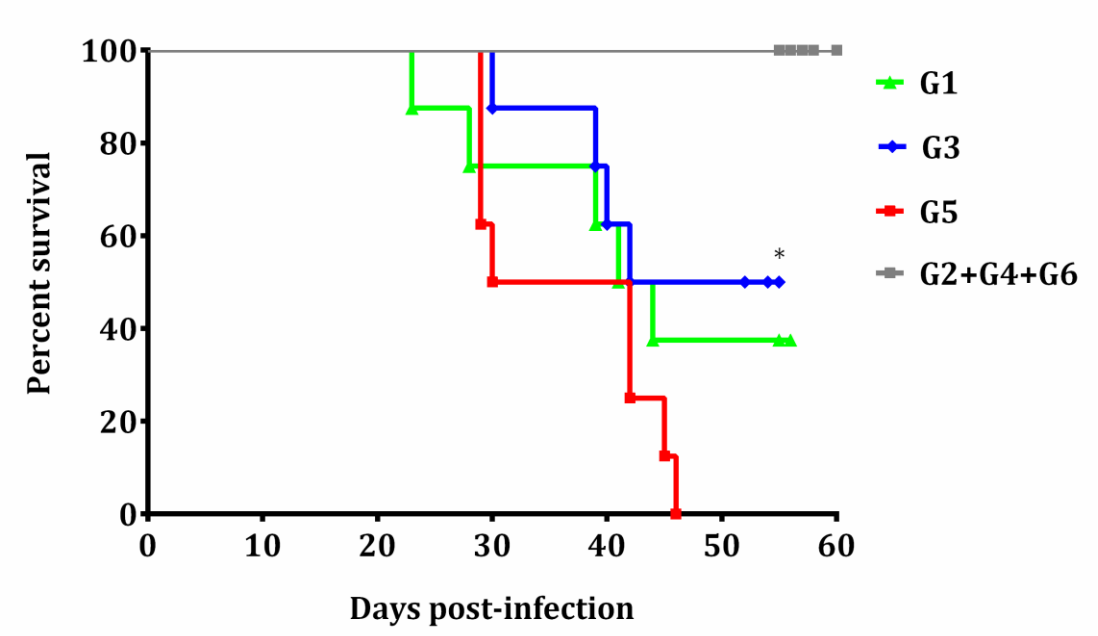


Table 2 - Haematological and biochemical parameters at initial and final time points.

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Parameter (units)	Reference values	G1		G2		G3	
		Initial	Final	Initial	Final	Initial	Final
Erythrocytes (x10^6)	9-14	10.99 ± 0.78	9.35 ± 1.09	10.70 ± 1.12	8.57 ± 0.83	10.49 ± 1.16	8.46 ± 1.40
Haemoglobin (g/dL)	8-15	12.31 ± 0.59	10.35 ± 1.22	12.10 ± 1.21	9.72 ± 1.13	11.75 ± 1.07	9.58 ± 1.31
Packed cell volume (%)	28-40	35.07 ± 2	30.48± 3.46	33.58± 3.31	27.66 ± 2.94	32.29 ± 2.54	26.73 ± 3.73
Platelets (x10^3)	250-750	498.50 ± 172.18	565.62 ± 186.80	442.20 ± 125.70	551.60 ± 207.95	476.50 ± 133.77	674.12 ± 255.49
Leukocytes (x10^3)	4-12	6.50 ± 0.83	5.33 ± 1.97	7.90 ± 1.76	6.98 ± 1.24	6.55 ± 1.12	6.90 ± 2.15
Segment neutrophils (%)	10-50	29.73 ± 4.46	35.63 ± 12.08	25.72 ± 7.81	32.32 ± 7.86	31.13 ± 7.64	36.27 ± 10.68
Lymphocytes (%)	40-75	60.01 ± 5.82	56.67 ± 10.69	63.16 ± 13.24	58.20 ± 9.84	59.31 ± 8.19	50.56 ± 8.77
Monocytes (%)	1-6	3.66 ± 1.25	2.78 ± 2.63	4.26 ± 1.75	3.80 ± 0.90	5.20 ± 2.44	6.91 ± 3.96*
Eosinophils (%)	0-15	5.37 ± 2.50	3.13 ± 1.05	5.62 ± 6.80	4.84 ± 2.06	3.04 ± 1.13	3.71 ± 2.35
Proteins (g/dL)	6-8	6.83 ± 0.63	6.08 ± 0.35	7.10 ± 0.86	6.18 ± 0.30	6.94 ± 0.71	6.52 ± 0.32
AST (UI/L)	70-210	72.75 ± 9.57	128.50 ± 15.40	131.40 ± 41.29	114.8 ± 26.78	76.88 ± 15.26	121 ± 46.03
GGT (UI/L)	36-93	67.50 ± 7.17	57.12 ± 7.98	83.80 ± 14.78	65.80 ± 11.43	73.13 ± 6.55	71.50 ± 10.05
ALP (UI/L)	44-355	370.37 ± 105.27	387.75 ± 257.94	482 ± 92.32*	680.20 ± 119.35**	326.88 ± 59.68	358.50 ± 130.59
CK (UI/L)	50-180	374.12 ± 108.24	251.62 ± 83.79	386 ± 60.02	226.60 ± 75.84	384.25 ± 83.03	314.12 ± 142.03
Urea (mg/dL)	8.4-30.8	15.60 ± 2.82	8.96 ± 2.31*	15.54 ± 0.71	10.20 ± 1.86	14.59 ± 4.15	13.73 ± 3.13
Creatinine (mg/dL)	0.9-1.7	0.96 ± 0.09	0.75 ± 0.10	1.02 ± 0.13	0.86 ± 0.05	0.94 ± 0.11	0.77 ± 0.07
Calcium (mg/dL)	7.1-9.8	9.92 ± 0.77	9.91 ± 0.62	9.86 ± 0.87	9.88 ± 0.43	9.61 ± 0.70	9.73 ± 0.48
Phosphorus (mg/dL)	3.5-7.3	7.11 ± 1.03	6.50 ± 0.75	6.62 ± 1.66	6.06 ± 0.87	6.59 ± 1.05	6.17 ± 0.56
Sodium (mEq/L)	139-152	150.37 ± 6.18	143.5 ± 1.60	149.80 ± 6.72	143 ± 3.08	148.25 ± 3.10	145.25 ± 1.90
Potassium (mEq/L)	3.9-5.2	5.21 ± 0.22	4.91 ± 0.15	5.12 ± 0.32	4.88 ± 0.29	5.15 ± 0.34	4.92 ± 0.33

Table 2 - Continued.

Parameter (units)	Reference values	G4		G5		G6	
		Initial	Final	Initial	Final	Initial	Final
Erythrocytes (x10^6)	9-14	10.62 ± 1.04	9.54 ± 0.59	9.76 ± 0.55	8.94 ± 0.74	10.81 ± 0.93	9.46 ± 0.44
Haemoglobin (g/dL)	8-15	11.56 ± 1.09	10.70 ± 0.59	10.79 ± 0.58	10.3 ± 0.76	11.83 ± 1.20	10.96 ± 0.90
Packed cell volume (%)	28-40	33.24 ± 2.56	30.52 ± 1.07	31.70 ± 2.39	29.63 ± 3.11	33.73 ± 3.37	30.33 ± 1.62
Platelets (x10^3)	250-750	402 ± 92.32	562.80 ± 146.51	470 ± 131.31	584 ± 150.43	440.33 ± 83.97	447.66 ± 48.08
Leukocytes (x10^3)	4-12	6.96 ± 1.49	6.91 ± 1.16	7.58 ± 1.98	5.69 ± 1.29	7.38 ± 3.28	7.03 ± 2.39
Segment neutrophils (%)	10-50	27.10 ± 7.03	33.24 ± 2.93	31.11 ± 7.71	24.12 ± 3.96	24.90 ± 8.57	29.86 ± 6.52
Lymphocytes (%)	40-75	63.48 ± 6.88	57.86 ± 3.51	58.94 ± 9.37	66.48 ± 4.85**	65.53 ± 9.43	59.40 ± 9.24
Monocytes (%)	1-6	4.24 ± 1.56	3.78 ± 0.17	3.78 ± 1.80	5.20 ± 2.20	3.40 ± 0.60	3.90 ± 1.38
Eosinophils (%)	0-15	3.86 ± 1.45	3.08 ± 1.25	4.94 ± 2.99	2.13 ± 0.48	4.90 ± 0.95	5.30 ± 3.17
Proteins (g/dL)	6-8	6.52 ± 0.33	6.32 ± 0.31	7.33 ± 0.91	6.31 ± 0.19	6.23 ± 0.45	6.46 ± 0.35
AST (UI/L)	70-210	79.60 ± 6.80	65.20 ± 4.76	92.13 ± 18.21	192.75 ± 49.41**	75.33 ± 10.59	68.66 ± 6.11
GGT (UI/L)	36-93	64.40 ± 2.30	67.20 ± 7.91	62.38 ± 26.25	70.12 ± 15.19	56.33 ± 10.59	57.33 ± 7.57
ALP (UI/L)	44-355	457 ± 231.50	423 ± 212.15	364 ± 91.51	247.75 ± 61.93	448 ± 142.67	553.66 ± 244.96
CK (UI/L)	50-180	423 ± 147.85	321.8 ± 96.69	376.38 ± 79.22	396.75 ± 194.55	363.67 ± 158.79	313.33 ± 198.04
Urea (mg/dL)	8.4-30.8	15.94 ± 3.71	12.48 ± 2.46	15.89 ± 2.73	13.18 ± 3.83	12.73 ± 4.85	12.60 ± 1.12
Creatinine (mg/dL)	0.9-1.7	0.90 ± 0.07	0.78 ± 0.04	0.93 ± 0.10	0.86 ± 0.10	0.83 ± 0.11	0.86 ± 0.05
Calcium (mg/dL)	7.1-9.8	9.82 ± 0.38	10.14 ± 0.39	8.94 ± 3.32	10.75 ± 0.37*	9.87 ± 0.25	10.63 ± 0.25
Phosphorus (mg/dL)	3.5-7.3	6.02 ± 0.27	5.76 ± 0.75	6.35 ± 0.82	6.12 ± 1.01	5.63 ± 0.61	5.30 ± 0.26
Sodium (mEq/L)	139-152	152.20 ± 13.33	145.60 ± 0.54	158.88 ± 13.27	144.87 ± 1.35	148 ± 1.73	145 ± 0
Potassium (mEq/L)	3.9-5.2	5.12 ± 0.21	5.14 ± 0.21	4.94 ± 0.39	4.80 ± 0.26	4.67 ± 0.05	4.80 ± 0.10

Values are represented as Means ± S.D. (*), $P < 0.05$ and (**), $P < 0.01$, indicating significant differences between groups at each time point for each parameter.

Biochemical parameters in the serum such as total proteins were not different from each other in all groups. Regarding liver function parameters, AST showed a significant increase ($P < 0.01$) in G5 compared to the other groups at the final time point, but was still within the normal range. GGT levels were in the physiological range in all groups, ALP mean values were above the normal range in most cases, and in G2 significantly higher values were noted at initial ($P < 0.05$) and final ($P < 0.01$) time points compared to the other groups. Concerning renal function parameters, urea levels were normal in all groups, but statistically lower in G1 compared to G3 at the final time point ($P < 0.05$). For all groups creatinine showed mean values within a normal range at initial time points, but these values decreased to below the normal range at final time points. CK, an early marker of myocardial damage, was 2-fold higher, thus above the normal physiological range in sheep initially and at the final time points in all groups. Levels of phosphorus, sodium and potassium were in the physiological range in all groups throughout the experiment, whereas calcium levels were slightly above the physiological range, and statistically elevated levels were noted in G5 at the final time point compared to the other groups ($P < 0.05$).

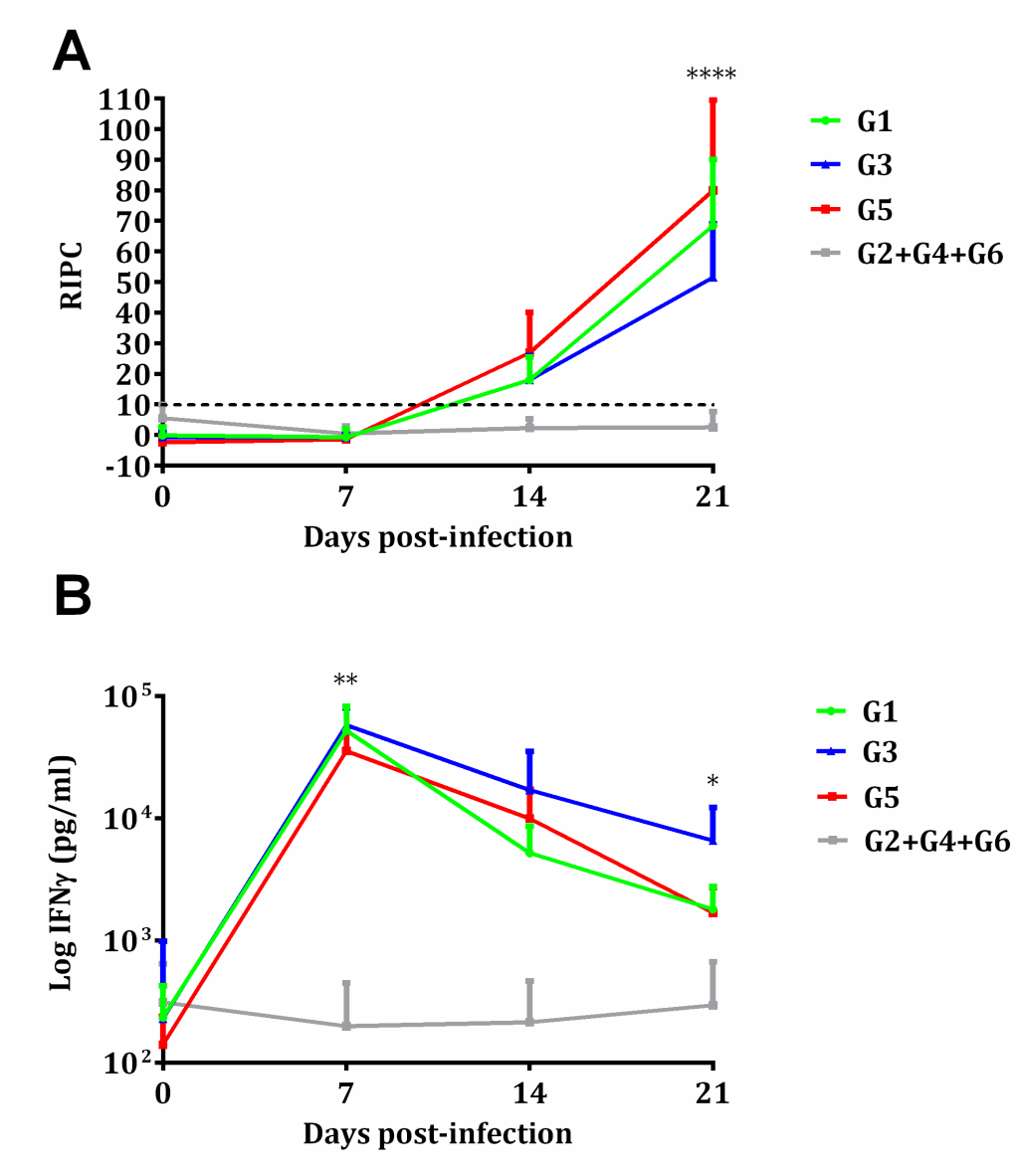
3.1.4. Humoral and cellular immune responses

The *N. caninum*-specific IgG antibody responses in dams analysed by ELISA are shown in Figure 3A. All uninfected control animals in G2, G4 and G6 exhibited basal IgG levels within the reference range throughout the experimental study. In contrast, compared to the control groups (G2 + G4 + G6), IgG levels increased significantly from day 14 pi in G5 ($P < 0.05$), and continued rising until day 21 pi, whereas in G1 and G3, significantly different IgG levels were only found from day 21 pi ($P < 0.0001$). On day 21 pi, G3 exhibited significantly lower IgG levels compared to G1 ($P < 0.05$) and G5 ($P < 0.0001$). On day 21 pi, IgG levels were also compared between aborted ewes and ewes that gave birth. No significant differences were found in G3, but lower IgG levels were observed in ewes that gave birth in G1 compared to those that aborted ($P < 0.01$). From day 21 pi until foetal death/birth occurred, IgG levels in G1 and G3 remained at similar values, but significantly lower values were noted in these two groups in relation to G5 ($P < 0.0001$) (data not shown).

Aborted foetuses in G1, G3 and G5 were all similarly seropositive, with titres ranging from 1:32 to 1:1024. Median values of the IFAT titres were calculated as 1:128 for G1 and G3, whereas G5 showed a median IFAT titre of 1:256. Precolostral sera collected from lambs born in G1 and G3 yielded positive titres ranging from 1:200 to 1:6400, with no significant differences, and median IFAT titres of 1:1600 in both groups (Additional file 1). Specific IgG responses against parasite antigen were not detected in foetuses/lambs from the three non-infected control groups (G2, G4 and G6).

IFNγ levels in supernatants of blood cell cultures recovered 24 hours after *N. caninum* antigen stimulation were significantly increased in samples from G1, G3 and G5 isolated on day 7 pi ($P < 0.0001$) and in cultures from G3 on day 14 pi ($P < 0.001$). In contrast, blood cell cultures from non-infected control animals (G2, G4 and G6) showed IFNγ levels that corresponded to the basal levels recorded prior to inoculation throughout the entire experimental study. The increased IFNγ levels observed in G1, G3 and G5 culture supernatants decreased from day 21 pi onwards and remained at low levels. Comparisons between infected groups showed significantly increased IFNγ levels on day 7 pi in G1 and G3 cultures compared to G5 ($P < 0.01$) and on day 14 pi in G3 compared to G1 ($P < 0.05$) (Figure 3B). When IFNγ values of stimulated cultures from aborting and non-aborting ewes were

Figure 3 - IgG response in sera (A) and IFNγ in supernatants of peripheral blood cell cultures (B). Values from infected (G1, G3 and G5) and uninfected (G2+G4+G6) pregnant ewes are represented. Each point represents the mean + S.D. at the different sampling times for each group. Data beyond day 21 pi are not shown, since several animals did not maintain pregnancy and were therefore sacrificed. Sera levels of total IgG antibodies against *N. caninum* are expressed as a relative index percent (RIPC), according to the formula: RIPC = (OD405 sample – OD405 negative control)/(OD405 positive control – OD405 negative control) × 100. Concentrations of IFNγ are expressed in pg/mL. Humoral and cellular immune responses represented in the figure were analysed using two-way ANOVA of repeated measures. For significant differences between infected groups, (*) indicates $P < 0.05$, (**) indicates $P < 0.01$ and (****) indicates $P < 0.0001$.



compared, no significant differences on day 7 pi were found in G1, but in G3 the cultures from aborting ewes produced significantly more IFNγ compared to cultures from non-aborting ewes ($P < 0.01$).

3.1.5. Pathology and lesion quantification

No histopathological lesions were found in the uninfected and treated groups (G2 and G4) or in the uninfected and untreated group (G6) (Figure 4A-C). Foci of necrosis with variable degrees of infiltration of inflammatory cells, primarily lymphocytes and macrophages, were found in all placentomes from all aborted ewes in infected groups (Figure 4B). Cotyledons from ewes that gave birth were too autolytic to permit proper histological evaluation. In foetal brains from infected sheep, lesions were found in 79%, 83% and 92% in G1, G3 and G5, respectively. In aborted foetuses from G1 and G3, 100% of foetal brains exhibited lesions, whereas 57% and 75% of foetal brains from lambs born in G1 in G3 showed lesions, respectively. Histological lesions were characterized by necrotic glial foci with random distribution in the neuropile (Figure 4D). The number of foci, average area of the lesions, and the percentages of damaged area showed no significant differences between infected groups (Additional file 1). However, within G1 and G3, when comparing the lesions found in aborted foetuses with those found in lambs, significant differences were found regarding the number of foci and percentage of damaged area. In G1, foetal brain from lambs born showed lower numbers of foci ($P < 0.05$) and percentages of damaged area ($P < 0.01$) compared to aborted foetuses. Similarly, foetal brains from lambs born in G3 showed lower numbers of foci ($P < 0.01$) and percentages of damaged area ($P < 0.05$) compared to aborted foetuses (Figure 5). No differences in those parameters were seen when aborted foetuses from different groups were compared, and when lambs from different groups were analysed.

Figure 4 - Hemotoxylin and eosin staining. A) Interdigitate area of the placentome of an uninfected sheep with no evident lesion. B) Foci of necrosis and scant inflammatory infiltration at the interdigitated area of the placentome of an infected sheep. C) Foetal brain from an uninfected sheep with no evident lesion. D) Glial foci with central necrosis at the foetal brain from an infected sheep. Bar: 200 μm.

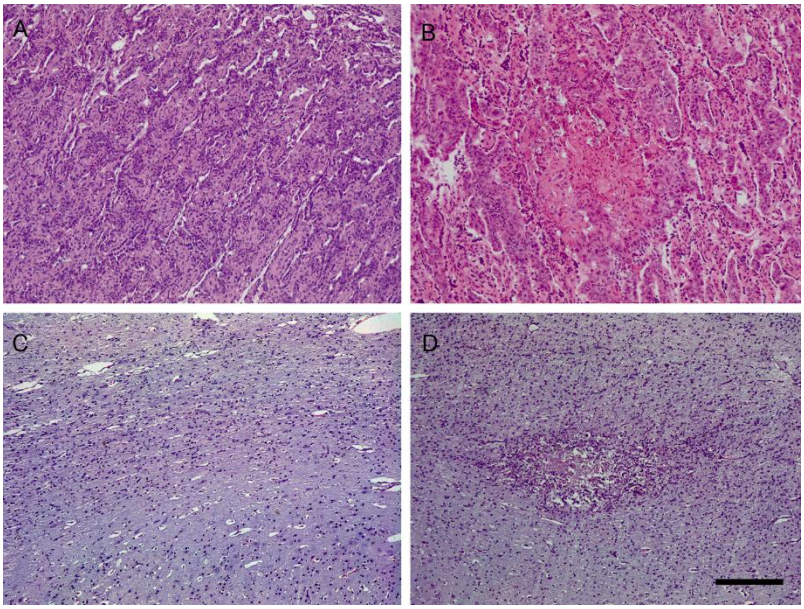


Figure 5 - Box-plots showing number of lesions (A), average size of focus (B) and lesion rates (C) in foetal brains from G1 and G3. Graphs represent the median percentage, the lower and upper quartiles (boxes) and minimum and maximum values (whiskers). Histological measurements of lesions were analysed using the non-parametric Kruskal–Wallis test followed by Dunn's test for comparisons between groups, as well as the Mann–Whitney test for pairwise comparisons. For significant differences between infected and treated groups, (*) indicates P < 0.05 and (**) indicates $P < 0.01$.

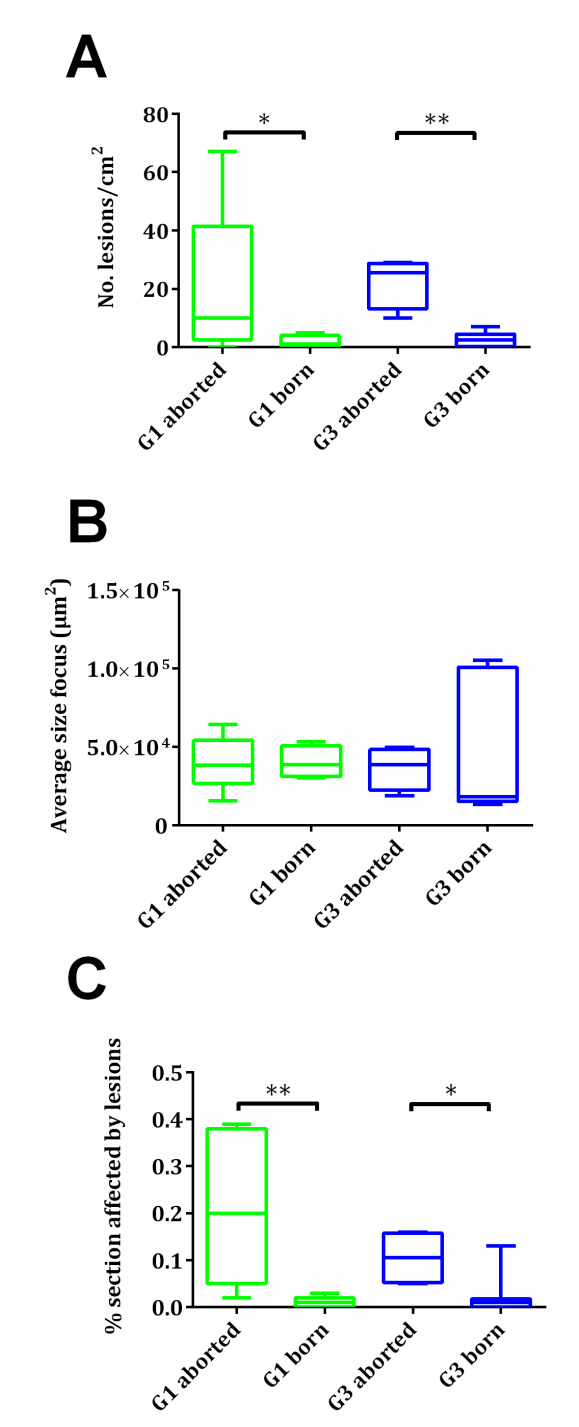
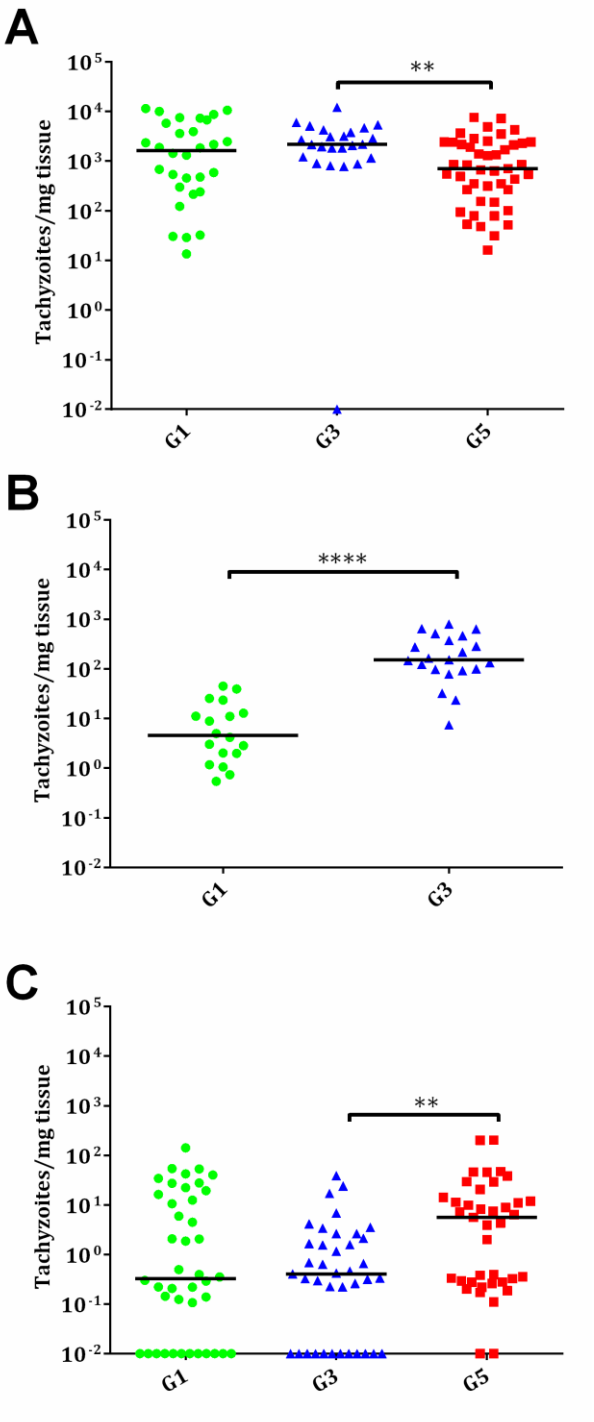


Figure 6 - Dot-plot graphs of *N. caninum* burdens in placentomes (A), cotyledons (B) and foetal brain (C) from G1, G3 and G5. Each dot represents individual values of parasite burden (number of parasites per mg of host tissue), and medians are represented as horizontal lines. Considering that the *N. caninum* detection limit by real-time PCR is 0.1 parasites, negative samples (0 parasites) were represented on the log scale as <0.1 (i.e., 10−2). Parasite burdens were analysed using the non-parametric Kruskal–Wallis test followed by Dunn's test for comparisons between groups, as well as the Mann–Whitney test for pairwise comparisons. For significant differences between infected groups in each tissue, (**) indicates $P < 0.01$ and (****) indicates $P < 0.0001$.



3.1.6. Parasite detection and burden in placental tissues and foetal brain

Neospora DNA was detected in placentomes or cotyledons from all ewes in the three infected groups, with 100% positive samples of the placentomes (30/30) or cotyledons (18/18) in G1, 96% positive samples of the placentomes (23/24) and 100% positive samples of the cotyledons (24/24) in G3 and 100% positive samples of the placentomes (48/48) in G5, with no statistical significances between them. The mean parasite burden (measured as the number of tachyzoites per mg of tissue) in placentomes from aborting ewes in G3 was higher compared to G5 ($P < 0.01$), and no significant differences were found compared to G1 (Figure 6A). Likewise, parasite burdens in cotyledons from ewes that gave birth were significantly lower in G1 than G3 ($P < 0.0001$) (Figure 6B).

A significantly higher percentage of *Neospora* positive foetal brains was detected in G5 (37/39) compared to G1 (30/42) ($P < 0.05$) and G3 (27/39) ($P < 0.05$) (Additional file 1). In G1 and G3, no significant differences were found for the percentage of detection in foetal brains between aborted foetuses and lambs born. In aborted foetuses of G1, a significantly higher number of foetal brain samples (19/21) was *Neospora* PCR positive compared to samples obtained from lambs born (11/21) ($P < 0.05$). However, no significant differences were found when comparing aborted foetuses (10/12) and lambs born (17/27) in G3. Furthermore, the overall parasite burden in the brains of aborted foetuses in G5 was significantly higher compared to G3 ($P < 0.01$) (Figure 6C) (Additional file 1). When comparing brain parasite burdens between infected groups, no significant differences were found in foetal brains between aborted foetuses or between lambs born. However, aborted foetuses in G5 showed higher brain parasite loads than lambs born from G1 ($P < 0.0001$) and G3 ($P < 0.001$). Additionally, significant differences were observed between aborted foetuses and lambs born in G1 when comparing brain parasite burdens ($P < 0.0001$). As expected, all placental and foetal samples from G2, G4 and G6 were negative.

3.2. Experiment 2: BKI-1553 plasma levels in foetal blood

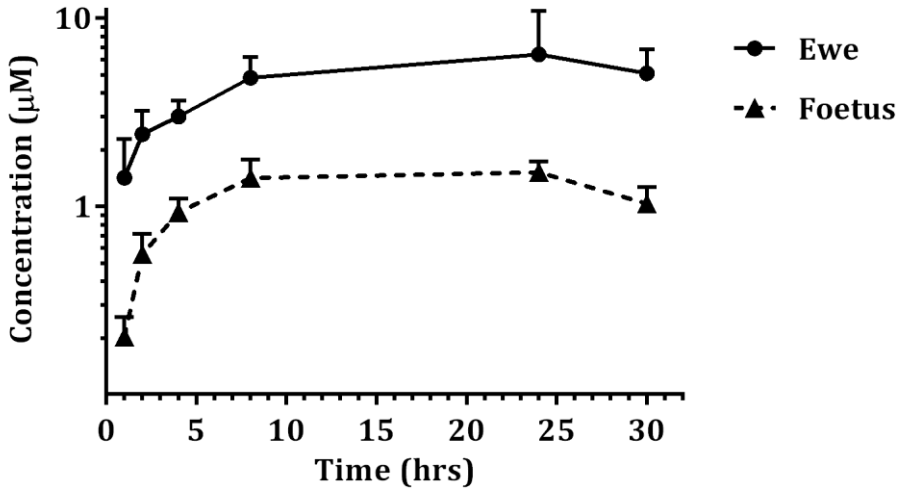
The C_{max} was 5.9 ± 3.2 μM for pregnant ewes and 1.6 ± 0.2 μM for foetuses, resulting in a ratio between foetuses and pregnant ewes of 0.27 ± 0.10 (Figure 7). The AUC was 2608 ± 1469 μM*min for pregnant ewes and 603 ± 61 μM*min for foetuses, resulting in a ratio between foetuses and pregnant ewes of 0.23 ± 0.12.

4. Discussion

This study reports on BKI-1553 drug levels in plasma of pregnant sheep and foetuses, and the safety and anti-parasitic efficacy of BKI-1553 treatment in a pregnant sheep model of neosporosis. The efficacy was assessed with respect to the clinical course of disease, immune responses, lesion development and parasite detection and load in placental tissues and foetal brains. To our knowledge, this is the first evaluation of a therapeutic drug candidate in a pregnant ruminant model of neosporosis.

Compared to previously developed BKIs, BKI-1553 has shown greatly improved bioavailability based on mouse pharmacokinetic data, with systemic levels >30-fold higher than BKI-1294 (Vidadala *et al.*, 2016). Consequently, BKI-1553 has been tested in calves, resulting in successful systemic exposure after a single dose with maximum concentrations 12 hours after administration (Schaefer *et al.*, 2016; Vidadala *et al.*, 2016), or multiple-doses with steady state plasma concentrations 25-fold higher than BKI-1294 (Schaefer *et al.*, 2016). Here, BKI-1553 demonstrated excellent exposure in pregnant ewes after multiple subcutaneous

Figure 7 - Plasma concentrations of BKI-1553 in pregnant ewes and foetuses after single 10 mg/kg subcutaneous dose. Each point represents the mean + S.D. at the different sampling times for pregnant ewes and foetuses.



applications. Maximum concentrations > 5 µM were reached at 12 hours after administration. Trough concentrations of approximately 4 µM were found, with > 1 µM in most of the pregnant ewes until the end of the sampling period. Because neosporosis greatly affects the foetus, foetal blood levels of BKI-1553 was evaluated in pregnant ewes. BKI-1553 application resulted in foetal plasma concentrations of 1.6 ± 0.2µM, which corresponded to approximately 20-30% of the systemic exposure in pregnant ewes over 24 to 30 hours post dose. Different sheep breeds were used in experiments 1 and 2. However, since very similar C_{max} were found in both experiments when the drug was applied at 10 mg/kg, it is conceivable that potential differences associated with the use of different sheep breeds in the two experiments can be discarded. Previous studies addressing the *in vitro* efficacy of BKI-1553 against *N. caninum* reported a half-maximal *inhibitory concentration* (IC_{50}) of 0.18 ± 0.03 µM (Müller *et al.*, 2017b). Plasma concentrations of BKI-1553 in pregnant ewes and their foetuses were higher than the IC_{50} for *N. caninum*, perhaps indicating adequate exposure that could translate into good efficacy in the pregnant sheep model of neosporosis. We did not find a significant association between individual levels of BKI-1553 and the outcome of foetal infection. However, the number of animals used here are small, and the variability in outcome may have been more affected by the actual drug concentrations in target tissues, which were not assessed. The concentration of free BKI-1553 in target tissues probably determines efficacy, and >90% of BKI-1553 is plasma protein bound, suggesting the levels of free drug may not have been completely adequate to achieve 100% efficacy (Vidadala *et al.*, 2016). In addition, some target tissues, such as foetal brain, may not have obtained sufficient drug levels to achieve complete efficacy (see below).

BKI-1553 treatment in mice did not cause any adverse side effects leading to toxicity, and complete blood counts and serum biochemical profiles were within normal ranges, suggesting that the compound was safe. However, although no gross abnormalities upon necropsy were found, histological examination revealed inflammation in the spleen and liver in several treated mice (Vidadala *et al.*, 2016). When BKI-1553 was applied to pregnant mice daily

at 20 mg/kg bodyweight for 5 days, it caused high neonatal mortality; this did not occur after administration at 20 mg/kg bodyweight 3 times with one-day intervals after each drug treatment (Müller *et al.*, 2017b). Likewise, BKI-1553 was safe in calves, with no toxicity observed after administration (Schaefer *et al.*, 2016; Vidadala *et al.*, 2016). In the present study, dermal nodules were found 24 hours after drug application at the sites of administration in all treated dams. Similar dermal nodules were found after vehicle (70% Tween 80 and 30% Ethanol 96º) administration in sheep (data not shown), indicating that the dermal nodules could be formed due to the vehicle. A formulation comprised of 7% Tween 80, 3% ethanol 96º and 90% normal saline has been already used in mice for oral administration of BKI-1294 (Ojo *et al.*, 2013) and in rats for oral and intravenous administration of antimalarial drugs (Van Voorhis *et al.*, 2007). In this study, higher percentage of Tween 80 and ethanol 96º was chosen as drug vehicle to achieve drug concentration that would allow the volume be administered subcutaneously. The much higher percentages of Tween 80 and Ethanol 96º could have triggered the appearance of mild dermal nodules. Concerning systemic side effects, non-infected pregnant ewes treated with BKI-1553 (G2 and G4) exhibited an increase in rectal temperatures on days 1 and 2 post-treatment start in G2 and on day 1 in G4; there were no associated abortions, and dams gave birth to healthy lambs without significant decreased birthweights. Furthermore, no microscopic lesions were found in placental and foetal tissues examined from these groups.

The haematological assessments in G2 and G4 showed no alterations in red and white blood cell counts (RBCs and WBCs, respectively). In contrast, in the infected groups, a minor monocytosis was found in G3, and an increase in lymphocyte percentages (still within the normal range) was detected in G5 at the final time point. Thus, due to normal monocyte percentages in G5 after infection, the minor monocytosis found in G3 could have been derived from dermal nodules arising after 7 BKI-1553 administrations over short intervals. Additionally, it was suggested that monocyte levels increase during the recovery period during inflammation (Weiss and Perman, 1992). Biochemical parameters in G2 and G4 to evaluate liver function, such as GGT and AST, were consistently in the physiological range at all time points. ALT displayed irrelevant higher values in G2; increased concentrations normally appear during late pregnancy in sheep (Yokus *et al.*, 2006). The apparent lack of liver toxicity is important because liver metabolism is hypothesized to be predominant for BKI-1294 in mice (Ojo *et al.*, 2013) and might also occur for BKI-1553, since it is also based on the naphthalinyl-pyrazolopyrimidine scaffold. Despite only 1% of BKI-1294 being excreted in urine (Ojo *et al.*, 2013), renal function parameters are essential for toxicity evaluation. In this study, no remarkable abnormalities were found for urea and creatinine values in G2 and G4. CK showed levels above the physiological range, although increased concentrations can typically appear in late pregnancy (Yokus *et al.*, 2006). CK and AST values have been previously described as valuable for investigating cardiotoxicity of drugs in lambs (Ekici and Isik, 2011). In contrast to BKI-1294, which was shown to be a potent human ether-a-go-go-related gene (hERG) inhibitor, BKI-1553 does not have a hERG liability (Vidadala *et al.*, 2016). Myocardial damage in sheep was not identified, since the corresponding markers AST and CK were not altered in G2 and G4 after BKI-1553 administration. No notable disorders related to minerals were observed after BKI-1553 administration, and the observed calcium levels slightly above the basal range may have been due to calcium regulation disruption associated with foetal demands in late pregnancy (Kovacs and Kronenberg, 1997).

Initiation of BKI-1553 treatment 48 h after infection was scheduled based on experience

with BKI therapy in murine neosporosis (Winzer *et al.*, 2015; Müller *et al.*, 2017b) and toxoplasmosis models (Doggett *et al.*, 2014; Huang *et al.*, 2015; Müller *et al.*, 2017c). The pregnant sheep model of neosporosis with infection on day 90 of pregnancy had been standardized to the extent that all pregnant ewes aborted between 34-48 days pi (Arranz-Solis *et al.*, 2015b). In this experiment, pregnant ewes from G5 displayed the same pattern, although a different breed was used. Clinical observations in the BKI-1553 treated groups revealed a partial protection against abortion, as 37 and 50 percent of the pregnant ewes gave birth in G1 and G3 respectively, with statistically improved foetal survival rates and median foetal survival times in G3. However, lambs in group G3 exhibited lower birthweights compared to uninfected lambs, regardless of treatment, which indicates that infection, but not the treatment, impacted on the weight in this group. Rectal temperatures in pregnant ewes in G5 increased from day 4 to day 7 pi, likely as a consequence of tachyzoite multiplication and the first cycles of parasite replication in host tissues, similar to previously reported results in cattle and goats experimentally infected with the Nc-Spain7 isolate (Regidor-Cerrillo *et al.*, 2014; Porto *et al.*, 2016). The rectal temperatures from pregnant ewes in G1 were lower than G5 on days 5 and 6 pi, suggesting that the drug had an impact on parasite replication. A decrease in rectal temperatures has also been described in experiments testing toltrazuril against neosporosis in calves (Kritzner *et al.*, 2002) and monensin (Buxton *et al.*, 1988) or decoquinate (Buxton *et al.*, 1996) against toxoplasmosis in pregnant ewes. Additionally, since AST typically increases after *T. gondii* infection, indicating liver injury (Yeo *et al.*, 2016), the increased AST in G5 and not in G1 and G3 at the final time point might indicate that treated pregnant ewes exerted better control of *N. caninum* infection.

Analysis of the peripheral immune responses in pregnant ewes at different time points demonstrated an increase in IFNγ release in stimulated peripheral blood cultures from G5 obtained at day 7 pi, showing similar kinetics to IFNγ in sera (Arranz-Solís *et al.*, 2016). Several reports have shown that a Th1-biased immune response against *N. caninum* is required to control tachyzoite proliferation (Entrican, 2002; Innes, 2007). IFNγ levels were higher on day 7 pi in G1 and G3 and on day 14 pi in G3, compared to G5. *In vitro* studies showed that BKI-1553 exhibited parasitostatic rather than parasiticidal effects, and induced the formation of intracellular multinucleated complexes composed of multiple pre-zoites unable to separate and form tachyzoites, but remaining viable for extended periods of time (Müller *et al.*, 2017b). These multinucleated complexes exhibit increased tachyzoite specific antigen1 (SAG1) expression, and also increased expression of the bradyzoite marker BAG1, with an overall heavily distorted parasite ultrastructure (Winzer *et al.*, 2015; Müller *et al.*, 2017b). If such complexes are also formed *in vivo*, they are unlikely to evade immune responses, but would be increasingly exposed to antigen-presenting cells, which would then result in higher IFNγ levels in treated animals. Likewise, increased IFNγ levels in G3 on day 14 pi might be caused by extended BKI-1553 administration in this group, although IFNγ levels were not elevated in the corresponding uninfected group G4. The increased levels of IFNγ in G1 and G3 might have led to greater initial control of parasitaemia at the peripheral level, diminishing the numbers of parasites reaching and invading the placenta (Entrican, 2002; Innes, 2007).

Concerning humoral immune responses, the levels of IgG started to increase in G5 on day 14 pi, which was in accordance to previously described results (Arranz-Solís *et al.*, 2016). However, antibody responses in the treated groups were delayed. As explained above, lymphocyte counts showed an increase in G5 at day 13 pi, and it is likely that this also includes B cells. On day 21 pi, significantly decreased

IgG levels were found in G1 and G3, but G3 showed lower IgG values than the other two infected groups G1 and G5. Similarly decreased antibody responses have been previously reported during treatments of *N. caninum* infected calves with toltrazuril (Kritzner *et al.*, 2002) and monensin (Buxton *et al.*, 1988) or decoquinate (Buxton *et al.*, 1996) treatment trials against toxoplasmosis in pregnant ewes. In treated groups, the delay at day 14 pi and lower antibody responses from day 21 pi onwards may have been due to higher IFNγ levels in the early stage of infection, as previously described Lopez-Gatius *et al.* (2007) under natural conditions. Likewise, longer IFNγ responses in G3 could contribute to reduced IgG levels on day 21 in this group. In contrast, on day 21 pi, IgG levels from pregnant ewes that aborted in G1 were higher than those that gave birth, suggesting that higher antibody responses occurred in animals that aborted their foetuses, as previously described Almería *et al.* (2016).

At approximately mid-gestation in pregnant ewes, the foetal immune system is undergoing development, according to specific antibodies detected in foetal fluids (Arranz-Solis *et al.*, 2015b). While BKI-1553 treatment had a beneficial impact on offspring survival, the drug did not prevent transplacental transmission. All foetuses/lambs were seropositive as assessed by IFAT. However, lower median IFAT titres were found in aborted foetuses from the treated groups G1 and G3, which is indicative for decreased antigen stimulation and thus enhanced control of *N. caninum* infection. To quantify the efficacy of BKI-1553 against transplacental transmission of *N. caninum*, microscopic lesions, detection and burden of *N. caninum* in placental tissues and foetal brains were investigated. In placental tissues *N. caninum* was widely detected in infected groups, as well as lesions in all placentomes from aborting ewes. In foetal brains, known as a predilection site for *N. caninum* (Collantes-Fernández *et al.*, 2006), parasites were less abundant, and the percentage of foetal brains with histological lesions was reduced in the treated groups. This is likely due to the efficacious BKI-1553 concentrations present in the foetuses. These results are consistent with those from a pregnant mouse model of neosporosis, in which reduced transplacental transmission to offspring was accomplished (Müller *et al.*, 2017b). In the treated groups, histopathological analysis revealed a lower percentage of foetal brains with lesions, lower numbers of foci, and lower percentage of damaged areas in lambs that were born compared to aborted foetuses, which is in accordance to previously described studies (Macaldowie *et al.*, 2004). Additionally, the parasite burden in foetal brains was reduced in G3 but not in G1, and in G1, but not G3, a higher parasite load was found in aborted foetuses compared to lambs born. Thus, the treatment undergone in G3 appeared to show higher efficacy in terms of controlling brain infection in the offspring. In both G1 and G3, cerebral parasite loads in lambs born were found to be lower compared to cerebral parasite loads in aborted foetuses in G5. Central nervous system (CNS) penetration by BKI-1553 could possibly explain why, despite a lack of difference in systemic exposures, some foetuses aborted and others were protected. CNS penetration of BKI-1553 was previously found to be approximately 33% compared to plasma exposure in mice (Vidadala *et al.*, 2016). If this is similar in sheep, then BKI-1553 concentrations in foetal brains would be ≤ 2 fold above the *N. canimum* IC_{50} level at the troughs for all dose concentrations. Such rather low concentrations would most likely only offer incomplete protection at this infection site in foetuses.

In conclusion, BKI-1553 treatment in *N. caninum* infected dams resulted in decreased rectal temperature upon infection, triggered an increase in peripheral IFNγ levels and a reduction in IgG responses, and achieved a reduced abortion rate due to neosporosis. In foetuses, BKI-1553 treatment did not prevent vertical transmission, but partially alleviated

the effects of infection, by reducing lesions, parasite presence and parasite loads in foetal brains. In the light of these findings, BKI-1553 exhibits an excellent systemic exposure in pregnant ewes and their foetuses, a tolerable safety profile and confers partial protection against abortion and foetal dissemination of the parasite in a pregnant sheep model of neosporosis. However, the reduction in terms of parasite detection in foetal brain was only 25%, which indicates a rather low efficacy of this particular treatment regime. Further studies are necessary to explore efficacy of BKI-1553, by applying alternative formulations and using other routes of administration, drug dosages and dosing regimes. In addition, other members of the BKI class of compounds under development could be tested in the near future against ruminant neosporosis.

Competing interests

The authors declare that they have no competing interests.

Author contributions

IF, AH, KO, WVV and LMO conceived the study and participated in its design. RSS wrote the manuscript, with results interpretation and discussion inputs from IF, JRC, AH, MH, KR, LB, WVV and LMO. LMF selected the animals and executed the reproductive programme. PGL and JRC prepared the inocula and performed the infections. RSS, PGL, MR, JBM, MPD, MGH, ET, PC and JB participated in inoculation and clinical examination of animals, performed necropsies and sampling of the animals and performed haematological, biochemical and histopathological analyses. MH, GR, KR, RC, LB, KO and WVV determined the pharmacokinetics of the compound. RSS and PV performed PCR and qPCR analyses, serological assays, statistical analysis and interpreted the results. All authors read and approved the final manuscript.

Acknowledgements

We gratefully acknowledge Jacobo Sampere from the SALUVET group (Complutense University of Madrid, Spain), Teresa Navarro and José María González (University of Zaragoza, Spain), Isabel Ayala, Lucas Troya, Clara Colmenero, Dolores González, Natalia Peral, Verónica Pérez, Ignacio Gómez and Isabel Santiago from the Clinical Veterinary Hospital (Complutense University of Madrid, Spain) for their excellent technical assistance, and also Rama Subba Rao Vidadala and Dustin Maly (Department of Chemistry, University of Washington) for their assistance in further purification of the study drug. The Animal Experimentation Service (SEA) at the University of Zaragoza is acknowledged for providing their facilities to carry out the reproduction programme. Roberto Sánchez Sánchez is supported by a fellowship from the Spanish Ministry of Education, Culture and Sports (MECD), as a part of the Program of Training of University Teaching Staff (FPU, grant number FPU13/03438). Patricia Vázquez has a Juan de la Cierva-Formación post-doctoral contract (FJCI-2014-20982) from the Spanish Ministry of Economy and Competitiveness (MINECO). Andrew Hemphill is supported by the Swiss National Science Foundation grant (No. 310030 165782). This work was supported by the Public Health Service, National Institutes of Health, Bethesda, MD (grants R01 AI 111341 and R01 HD 080670), the U.S. Department of Agriculture (grant 2014-67015-22106) and the Community of Madrid, Spain (PLATESA, S2013/ABI2906).

Additional file 1 - Individual serological titres in foetuses/lambs, measurement of histological lesions and detection of parasite DNA and parasite load in foetal brain.

Group	Ewe ref.	Foetal death (days p.i)[a]	Foetus/lamb ref.	IFAT[b]	Foetal brain				
					Histopathology			DNA[f]	qPCR[g]
					NF[c]	ASF[d]	% Les[e]		
Group 1	1.1	28	1.1 F1	1:128	46	43130	0,39	+++	6.17 ± 5.24
			1.1 F2	1:128	67	35650	0,38	+++	38.23 ± 17
			1.1 F3	1:64	37	54159	0,37	+++	63.76 ± 51.53
	1.2	44	1.2 F	NA	9	64245	0,09	++	0.14 ± 0.13
	1.3		1.3 F1	1:3200	5	53386	0,03	+++	1.31 ± 0.97
			1.3 F2	1:1600	4	34239	0,02	+++	0.23 ± 0.18
	1.4		1.4 F1	1:1600	1	30191	0,01	++	0.06 ± 0.05
			1.4 F2	1:1600	0	0	0	+	0.08 ± 0.12
			1.4 F3	1:1600	0	0	0	-	0.01
	1.5		1.5 F	1:128	27	38173	0,2	+++	12.90 ± 11.64
	1.6		1.6 F1	1:1600	0	0	0	+	4.68 ± 8.10
			1.6 F2	1:256*	2	43278	0,01	+	0.15 ± 0.24
	1.7	41	1.7 F	NA	10	26710	0,05	++	0.18 ± 0.15
	1.8	23	1.8 F	1:64	3	15426	0,02	+++	27.13 ± 27.06
Group 3	3.1	42	3.1 F	1:512	28	49651	0.16	+++	0.35 ± 0.18
	3.2		3.2 F1	1:512*	7	105205	0.13	-	1.16 ± 1.99
			3.2 F2	1:6400	0	0	0	+++	2.02 ± 1.41
	3.3		3.3 F1	1:3200	0	0	0	+++	0.66 ± 0.65
			3.3 F2	1:3200	3	20212	0.01	++	0.70 ± 1.01
	3.4		3.4 F1	1:200	3	15901	0.01	++	1.66 ± 1.77
			3.4 F2	1:800	5	16094	0.01	++	6.33 ± 10.41
			3.4 F3	1:128*	NA	NA	NA	++	0.41 ± 0.46
	3.5	40	3.5 F	1:128	23	18814	0.05	++	0.18 ± 0.15
	3.6		3.6 F1	1:1600	2	99271	0.02	-	0.06 ± 0.10
			3.6 F2	1:200	1	13254	0.00	+	0.09 ± 0.13
	3.7	30	3.7 F	1:128	29	33035	0.15	+++	19.73 ± 12.76
	3.8	39	3.8 F	1:128	10	44310	0.06	++	1.05 ± 1.03

Additional file 1 - Continued.

Group	Ewe ref.	Foetal death (days p.i)[a]	Foetus/lamb ref.	IFAT[b]	Foetal brain				
					Histopathology			DNA[f]	qPCR[g]
					NF[c]	ASF[d]	% Les[e]		
Group 5	5.1	42	5.1 F	NA	21	21305	0.06	++	0.18 ± 0.15
	5.2	30	5.2 F	1:512	50	37769	0.38	+++	121.47 ± 72.90
	5.3	29	5.3 F1	1:128	27	40015	0.11	+++	0.16 ± 0.11
			5.3 F2	1:256	18	26902	0.13	+++	0.59 ± 0.71
			5.3 F3	1:256	2	47219	0.05	+++	16.28 ± 8.82
			5.3 F4	1:32	0	0	0	+++	8.67 ± 4.39
	5.4	46	5.4 F	1:512	1	24360	0.01	+++	0.23 ± 0.06
	5.5	29	5.5 F	1:512	20	42148	0.09	+++	6.82 ± 2.02
	5.6	45	5.6 F	1:1024	1	68851	0.05	+++	0.28 ± 0.02
	5.7	42	5.7 F1	NA	9	22249	0.08	++	1.85 ± 3
			5.7 F2	1:256	6	16883	0.03	+++	20.66 ± 19.23
	5.8	29	5.8 F1	1:64	27	30493	0.20	+++	37.53 ± 25.67
			5.8 F2	1:512	25	28526	0.18	+++	11.04 ± 10.69

[a] Day post-challenge when foetal death was detected by ultrasonography. The remaining foetuses lived until the end of the experiment.

[b] IFAT IgG antibody titres in foetal body fluids and in precolostral serum collected after birth in lambs born alive.

[c] Number of foci (number of lesions/cm^2).

[d] Average size focus (μm^2).

[e] Percentage of damaged area.

[f] Parasite DNA detection; plus (+++, ++, +) and minus (-) signs represent PCR detection in >67%, 66-34%, <33% and 0% of samples analysed, respectively.

[g] Mean parasite load (tachyzoites/mg tissue) and standard deviation (S.D.). Taking into account that the *N. caninum* detection limit by real-time PCR is 0.1 parasites, negative samples (0 parasites) were represented as 0.01.

* Lamb dead at birth.

NA: not available.

CAPÍTULO V

DISCUSIÓN GENERAL

GENERAL DISCUSSION

“La ciencia nunca resuelve un problema
sin crear otros 10 más”

George Bernard Shaw (1856-1950)

T. gondii and *N. caninum* are apicomplexan parasites considered to be the one of the main infectious causes of abortion in sheep (Dubey, 2009b) and cattle (Dubey and Schares, 2011), respectively. Thereby, toxoplasmosis and neosporosis deserve special attention since they entail severe economic losses in ovine (Innes *et al.*, 2009) and cattle population (Trees *et al.*, 1999; Reichel *et al.*, 2013), respectively. For the control of toxoplasmosis and neosporosis different measures have been suggested, including management practices, chemotherapy and vaccination (Dubey *et al.*, 2007; Dubey, 2009b). In bovine neosporosis, the use of drugs and vaccines are considered the best control strategies to avoid reactivation (Reichel and Ellis, 2006). However, in toxoplasmosis, the same tools should be preferable applied if there is a likely environmental contamination with oocysts or during an abortion outbreak to avoid the clinical manifestation of the disease. A commercial attenuated live vaccine based on a modified strain (S48), with no capacity to form tissue cysts and oocysts, is available for ovine toxoplasmosis, allowing an increase of around 60% in the number of live lambs (Buxton *et al.*, 1991). However, despite the great effort on developing a vaccine against neosporosis, there is no vaccine available in the market (Hemphill *et al.*, 2016; Horcajo *et al.*, 2016). Likewise, unfortunately, there are no drugs registered for the treatment of toxoplasmosis and neosporosis in ruminants (Sánchez-Sánchez et al., accepted for publication). Therefore, there is an urgent need to develop drugs and vaccines aimed at preventing their transmission, as well as reducing severity of these diseases. In this sense, a large amount of research focuses its efforts on the development of new candidates that may be effective in protecting against abortion and vertical transmission. For that purpose, both *in vitro* and *in vivo* models have served as steps in the process of testing safety and efficacy of new products. *In vitro* assays offer a reduction in the usage of animals for experimentation, and provide high throughput screening of drugs or antibodies directed against potential vaccine targets (Müller and Hemphill, 2013). However, animal models are essential for the study of several aspects that otherwise could not be assessed by means of *in vitro* culture. Apart from being of great utility for studies related to host-parasite relationship, immune response or pathogenesis, experimental animal models of infection are essential tools for an adequate evaluation of efficacy of vaccines or drugs developed against toxoplasmosis and neosporosis (Jongert *et al.*, 2009; Reichel and Ellis, 2009).

T. gondii presents three different clonal lineages classified as I, II and III (Howe and Sibley, 1995). Laboratory mice are generally sensitive to *T. gondii* infection and are often used as the preferred animal model to determine the virulence of the parasite (Saraf *et al.*, 2017). Type I and most South American isolates are highly virulent in mice whereas types II and III show a dose-dependent mortality (Saeij *et al.*, 2006). Enhanced virulence throughout successive passages in cell culture and mice for *T. gondii* type I isolates (RH isolate) has been widely reported (Villard *et al.*, 1997; Mavin *et al.*, 2004; Khan *et al.*, 2009b), however, little is known about influence of continuous passages in type II isolates. Likewise, mice have been widely used as animal model for congenital toxoplasmosis due similarities in placental histology of rodents and humans. In addition, mice offer several advantages such as the small size, ease of handling, low cost, short period of gestation and high number of pups. However, structure of the placenta, reproductive physiology and immune responses greatly differ between rodents and ruminants, which clearly could influence the passing of *T. gondii* trough the maternofetal interface and the modulation of the host immune responses during pregnancy (Entrican, 2002). In sheep, most of experimental studies during pregnancy have been carried out using M1, M3 and M4 *T. gondii* strains (Dubey, 2009b; Castaño *et al.*, 2014) and the congenital infection in mice of these strains has not been studied in depth.

On the other hand, despite the broad usage of the mice model for the study of neosporosis and for "proof-of-concept" assays of vaccine and drug candidates, the obvious physiological and size differences between rodents and ruminants render the results obtained in mice poorly reliable. In this

sense, the employment of other animal models of neosporosis more proximate to cattle could serve as a good alternative. Besides being a natural host of *N. caninum*, sheep offer several advantages over cattle in terms of number and size of animals, handling, costs and timing of experiments. Therefore, it would seem suitable as a model for further research. Moreover, even though the epidemiological, clinical and economic relevance of the parasite for reproductive failure in small ruminants has remained elusive, recent studies suggest that neosporosis may be a more important cause of reproductive disorders than it has traditionally been considered, at least in certain scenarios (West *et al.*, 2006; Howe *et al.*, 2012; Moreno *et al.*, 2012; González-Warleta *et al.*, 2014; Gonzalez-Warleta, submitted). In pregnant sheep, infective doses of 10^7–10^8 tachyzoites results in a high percentage of abortions (Dubey and Lindsay, 1990; Buxton *et al.*, 2001; Innes *et al.*, 2001a; Weston *et al.*, 2009) and there is only one study comparing different infective doses (Weston *et al.*, 2009). Likewise, to date, there are no studies comparing the outcome of *N. caninum* experimental infection using different routes of inoculation in pregnant sheep.

To date, pregnant ruminant models of neosporosis have not been used for assessments of drug efficacy against *N. caninum* infection and vertical transmission. In toxoplasmosis, drugs evaluated in pregnant sheep (monensin, folate inhibitors and decoquinate) showed moderate protection against abortion and limited or no protection against vertical transmission. Therefore, there is lack of effective drugs for the treatment of congenital toxoplasmosis and neosporosis in ruminants. Highly promising drugs candidates have been tested against *T. gondii* and *N. caninum in vitro* and in small animal models, such as thiazolides, diamidines, artemisinins, naphthoquinones, anticancer agents, endochin-like quinolones and calcium-dependent protein kinase inhibitors (Sánchez-Sánchez et al., accepted for publication). Calcium dependent protein kinase 1 (CDPK1) represents a promising drug target, as CDPK1 is encoded by the apicoplast DNA, and is thus absent from mammalian hosts (Lourido *et al.*, 2010; Murphy *et al.*, 2010; Ojo *et al.*, 2010) and it is conserved among apicomplexan parasites (Keyloun *et al.*, 2014). CDPK1 activity is essential for microneme secretion, host cell invasion, and egress of *T. gondii* (Kieschnick *et al.*, 2001; Lourido *et al.*, 2010) and can be effectively targeted by a class of ATP-competitive compounds, collectively named bumped kinase inhibitors (BKIs). The compound BKI-1294 was effective *in vitro* against *N. caninum* and *T. gondii* and showed highly protection against vertical transmission in mice models of congenital toxoplasmosis and neosporosis (Winzer *et al.*, 2015; Müller *et al.*, 2017c). Likewise, the compound BKI-1553, with an improved bioavailability compared to BKI-1294 in mice and calves (Schaefer *et al.*, 2016; Vidadala *et al.*, 2016) was also effective *in vitro* against *N. caninum* and *in vivo* in a pregnant mice model of neosporosis (Müller *et al.*, 2017b). However, the safety of BKI compounds in pregnant sheep and their efficacy in pregnant sheep models of toxoplasmosis and neosporosis are unknown.

Taking all of this into consideration, the present Doctoral Thesis addresses three main objectives: the standardization of pregnant mice and sheep models of *T. gondii* infection and the comparative assessment of virulence of *T. gondii* type II isolates in mice and sheep (Objective 1), the standardization of a well-defined pregnant sheep model of neosporosis (Objective 2) and the evaluation of safety and efficacy against *T. gondii* and *N. caninum* of BKIs in experimentally infected pregnant sheep (Objective 3).

Standardization of pregnant mice and sheep models of *T. gondii* infection and comparative assessment of virulence of *T. gondii* type II isolates in mice and sheep

For the attainment of the first objective, the phenotype *in vitro*, virulence in mice and congenital infection in mice and sheep were compared between a newly obtained *T. gondii* type II isolate (TgShSp1) and the laboratory type II reference isolate (TgME49).

In Europe and North America, *T. gondii* isolates display a clonal population structure, with the vast majority of *T. gondii* isolates being grouped into three lineages, namely, types I, II and III (Howe and Sibley, 1995). Type II *T. gondii* is the most prevalent in all hosts in Europe, including sheep (Dumètre *et al.*, 2006; Halos *et al.*, 2010a; Su *et al.*, 2010). Previous studies in Europe have shown that *T. gondii* type II is associated with ovine abortion (Owen and Trees, 1999; Jungersen *et al.*, 2002; Chessa *et al.*, 2014). The TgShSp1 isolate belongs to genotype #3 (a type II variant, II for nine alleles/I for Apico), sharing genotype with the Prugniaud (PRU) isolate.

T. gondii PRU isolates exhibit a similar genetic pattern to the *T. gondii* type II reference isolate, TgME49 (genotype #1) (Su *et al.*, 2012). In addition, both type II isolates, TgME49 and PRU, activate the host cell transcription factor NF-κB, an integral component of the immune response to *T. gondii*, and they display identical GRA15 gene sequences, which is involved in NF-κB activation (Rosowski *et al.*, 2011). TgME49 was isolated from sheep muscle in 1958 (Lunde and Jacobs, 1983) and has since then undergone long-term passaging in cell culture and mice (Sibley *et al.*, 2002). Previous studies have demonstrated changes in biological characteristics of *T. gondii* isolates after passages in mice and cell culture (Frenkel *et al.*, 1976; Lindsay *et al.*, 1991; Harmer *et al.*, 1996; Saraf *et al.*, 2017). This fact has been widely studied in *T. gondii* type I isolates (Cesbron and Sabin, 1994; Villard *et al.*, 1997; Dubey *et al.*, 1999; Mavin *et al.*, 2004; Khan *et al.*, 2009b). However, whether these changes also occur in type II isolates, and how they compare to recently obtained isolates, remains unknown. Increased growth *in vitro* can be found after repeated passages (Yano *et al.*, 1987). The dramatic differences observed during *in vitro* growth of TgME49 and TgShSp1 might reflect the highly different passage history of the two isolates. Plaque formation is commonly used to measure growth of *T. gondii*, and this process is the result of several events, including invasion, growth, egress, and migration (Roos *et al.*, 1995). Notably, TgME49 tachyzoites formed plaques at 4 days pi, but TgShSp1 did not. The finding that TgShSp1 did not form plaques *in vitro* could have resulted from the limited growth rate, associated with a higher capacity of bradyzoite conversion, as previously described Khan *et al.* (2009b). This was confirmed by monitoring spontaneous cyst formation through labeling with the fluorescent lectin DBL, demonstrating tissue cyst formation under standard cell culture procedures in TgShSp1 that was greater than TgME49. Due to its high passage number in cell culture or mice, our TgME49 isolate may not accurately represent natural virulence traits of the type II lineage, which suggests that comparisons of phenotypes between *T. gondii* isolates should be conducted using low-passage stocks.

Traditionally, mouse models are utilized to evaluate virulence by monitoring survival after experimental infection. Conventionally, TgME49 is a cystogenic type II isolate with low virulence in mice (Ferreira *et al.*, 2001; Gavrilescu and Denkers, 2001; Oliveira *et al.*, 2016). Intraperitoneal inoculation of 10^3 and 5 x 10^4 TgME49 tachyzoites intraperitoneally has not caused mortality (Ferreira *et al.*, 2001; Oliveira *et al.*, 2016). However, in this study, TgME49 displayed a LD_{50} of 10^3, so the virulence of our TgME49 could be considered higher than previous descriptions (Ferreira *et al.*, 2001; Oliveira *et al.*, 2016). Enhanced virulence in mice for *T. gondii* strains maintained for several passages has also been reported previously (Shimizu *et al.*, 1967; Sibley and Boothroyd, 1992; Frenkel and Ambroise-Thomas, 1996). Hence, it seems logical to speculate that results from studies using laboratory isolates should be validated with more recent isolates before they can be extrapolated as general features of the respective lineage. In contrast to TgME49, mice inoculated with tachyzoites of the recently obtained type II isolate TgShSp1 exhibited only moderate, low-level clinical signs, but no mortality ($LD_{50} > 10^5$), similar to what was described earlier after intraperitoneal inoculation of Swiss Webster mice with 10^3 tachyzoites of a PRU isolate (Wang *et al.*, 2013). In addition, although oocysts are considered more virulent than tachyzoites in mice (Dubey and Frenkel, 1973), no

mortality in adult mice was found after infection with oocysts of TgShSp1 in pregnant and non-pregnant mice, suggesting very low virulence in mice.

We also investigated congenital toxoplasmosis in pregnant mice by inoculating them orally with different doses of TgShSp1 oocysts. The risk of congenital toxoplasmosis depends on the virulence of the parasite (Tenter *et al.*, 2000). Few mice infected with TgShSp1 oocysts showed mild clinical signs, contrary to the large number of mice succumbing to infection after the same oocyst doses of TgME49 (Müller *et al.*, 2017c). There is a possibility that there has been a selection towards increased virulence within TgME49 due to the successive passages, as explained above but also due to the sulfadimidine treatment that was applied in mice used for infection of cats to generate TgME49 oocysts (Müller *et al.*, 2017c). This sulfonamide treatment could act as a bottleneck in selecting tachyzoites with a faster replication and therefore increasing the virulence in mice of the final TgME49 parasites. Unlike what was observed for TgME49 (Müller *et al.*, 2017c), with which a clear effect on pregnancy rate was found after infection with 2000 oocysts and on litter size after infection with 500 oocysts, infection with TgShSp1 oocysts did not result in alteration of pregnancy rate or litter size. Likewise, while 92% of the pups died after infection of dams with 25 TgME49 oocysts (Müller *et al.*, 2017c), a significant decrease in pup survival was found only after infection with 2000 and 500 TgShSp1 oocysts, where there was mortality in 50% of the pups.

In sheep, although rectal temperatures were similarly increased in TgME49 and TgShSp1, ewes infected with 500 and 10 TgME49 oocysts exhibited higher rectal temperature on day 8 pi compared to same doses of TgShSp1 oocysts. Perinatal mortality was similar for both isolates. However, those ewes infected with 10 and 50 TgME49 oocysts and that delivered stillbirths/live lambs exhibited higher parasite load in cotyledons than those infected with the same doses of TgShSp1 oocysts. Similarly, a higher parasite load was found in the brain from lambs born in the group infected with 50 TgME49 oocysts compared to the corresponding TgShSp1 group. Therefore, it is tempting to hypothesize that the enhanced virulence of our TgME49 contributed to the abovementioned effects. Comparing different doses of infection, pregnant ewes challenged with 50 and 10 oocysts showed one day of delay in the increase of rectal temperature compared to ewes infected with 500 oocysts in both isolates, similar to what was described by Buxton *et al.* (1991) and Mévélec *et al.* (2010). Likewise, there is a correlation between the dose of infection and the rate of early abortions, as previously suggested (Mévélec *et al.*, 2010; Benavides *et al.*, 2017). Infection with 500 oocysts triggered abortion in all fetuses, similar to previous experimental infections in pregnant sheep at mid-pregnancy using 2000 oocysts of M1 and M4 isolates (Owen *et al.*, 1998a; Castaño *et al.*, 2014). After infection with 50 TgShSp1 oocysts or 50 TgME49 oocysts, 68 and 42% of fetuses/lambs died, respectively, similar to what was previously reported after infection with 50 M4 oocysts (Castaño *et al.*, 2014). The occurrence of abortions after infection with 10 oocysts was low, but large numbers of stillbirths and weak lambs were found, mainly in the 10 TgShSp1 oocysts group. There seems to be a correlation between the presence of the parasite and the occurrence of stillbirths, since stillbirths from the group infected with 10 TgShSp1 oocysts exhibited higher parasite detection and load in the brain than those in the group infected with 50 TgShSp1 oocysts. Regardless of the isolate or dose, no differences were found in the congenital infection of lambs born, since vertical transmission was found in all them except in one stillborn lamb and one live lamb born from one ewe infected with 10 TgME49 oocysts. Likewise, no differences were found between doses or isolates with respect to brain lesion presence and lesion severity in lambs born.

Despite the clear differences in body weight between mice and sheep, similar doses of oocysts were used to compare both hosts. None of the adult mice infected with 25 TgShSp1 oocysts exhibited clinical signs, whereas all ewes infected with 10 TgShSp1 oocysts had fever. Therefore, morbidity in

sheep seems to be higher than in mice. In addition, no mortality was observed in adult mice or sheep infected with TgShSp1 oocysts. When comparing the congenital infection between both hosts, 50% mortality was caused in mice by infection with 500 TgShSp1 oocysts, whereas in sheep infection with the same oocyst dose caused mortality in all fetuses. In brief, mice seem to be less susceptible to perinatal mortality than sheep, despite the fact that vertical transmission was similar in both species. High vertical transmission and low offspring mortality could be an evolutionary strategy of the parasite to generate a large infected offspring group in mice, one of the most relevant hosts of *T. gondii* (Müller and Howard, 2016).

There are several differences between mice and sheep that could underlie the differences found in this study. The histological structure of the placenta is very different between mice and sheep (Entrican, 2002), and although maternal blood and fetal tissue are closer in mice, allowing an easy crossing of tachyzoites but also of antibodies, the longer period of gestation, the lack of maternal antibodies crossing the placental barrier and fewer fetuses may facilitate vertical transmission in sheep. In addition, host genetics are likely important in determining susceptibility and severity of infection (Howe *et al.*, 1996; Müller and Howard, 2016). Small rodents, natural intermediate hosts, are often exposed to a higher dose and more virulent parasites than other groups of mammals. It may therefore be that Toll-Like-Receptors (TLR)11 and TLR12 and the polymorphism of immunity-related GTPases (IRG proteins) have been positively selected in rodents, because of their critical importance in host resistance against high infection loads or more virulent clones of *T. gondii*. In mice, TLR11 and TLR12 on dendritic cells detect the apicomplexan actin-binding protein profilin leading to the secretion of interleukin 12 (IL12), which can subsequently induce production of IFNγ by T cells. IFNγ induces a variety of parasiticidal mechanisms, which in mice are dominated by upregulation of the IRGs. IRGs can destroy the vacuole in these parasites live and subsequently the parasite itself. Considering the ubiquity of *T. gondii* in nature, it is intriguing that genes encoding TLR11, TLR12, and IRG proteins are not found in many mammalian species (Gazzinelli *et al.*, 2014). Although further studies are needed, and despite the influence of genetic polymorphisms in ovine abortions (Darlay *et al.*, 2011), this fact could render sheep less resistant. Similarly, differences in immune cell populations may influence the pathogenesis of toxoplasmosis in these hosts. γδ T cells, which rapidly recognize and respond to nonprocessed antigens and seem to have an important role in *T. gondii* infection (Egan *et al.*, 2005), represent a relevant subset of circulating T cells in sheep compared to mice (Holderness *et al.*, 2013). Further studies are needed to characterize the cellular and molecular bases contributing to transmission dynamics and disease in different hosts of *T. gondii* (Dubremetz and Lebrun, 2012; Hunter and Sibley, 2012).

In conclusion, we have demonstrated that infection with tachyzoites and oocysts of the type II *T. gondii* isolate TgShSp1 in mice does not cause mortality, but this isolate is efficiently vertically transmitted in pregnant mice, and compared to sheep, it triggers lower offspring mortality and morbidity. Thus, at least for this isolate, the disease caused in pregnant mice and offspring is not a reliable predictor/indicator for disease caused in pregnant sheep at mid-gestation. Whether this conclusion is also valid for other type II *T. gondii* strains needs to be addressed in future studies. Because sheep have neither TLR11/12 nor the IRGs and are reported to express the "human" rather than the "murine" type of TLR members (Nalubamba *et al.*, 2007), we hypothesize that sheep can serve as a better animal model to study *Toxoplasma*-host interactions that are relevant for humans and farm animals.

Standardization of a well-defined pregnant sheep model of neosporosis

Regarding the pregnant sheep model of neosporosis, there is only one study comparing different infective doses (Weston *et al.*, 2009) and, to date, there are no studies comparing the outcome of *N. caninum* experimental infection using different routes of inoculation in pregnant sheep. Consequently, the second objective of the present Doctoral Thesis was to optimise the challenge dose of *N. caninum* and route of administration in pregnant sheep and, therefore standardize the pregnant sheep model of neosporosis for its use in further studies. For the attainment of the second objective, a dose titration of *N. caninum* tachyzoites by the intravenous route of administration as well as an evaluation of the subcutaneous route of administration were carried out in pregnant sheep at mid-pregnancy by assessing clinical course of disease, cellular and humoral immune responses, lesion development and parasite detection and burden in placental and foetal tissues. The Nc-Spain7 isolate (Regidor-Cerrillo *et al.*, 2008) used in this experiment is a very well-characterized virulent isolate tested so far in three experimental ruminant models, such as sheep (Arranz-Solis *et al.*, 2015b), goats (Porto *et al.*, 2016) and cattle (Caspe *et al.*, 2012; Regidor-Cerrillo *et al.*, 2014; Almería *et al.*, 2016). Since intravenous infection of pregnant sheep using 10^6 tachyzoites of the Nc-Spain7 isolate at mid-gestation resulted in 100% abortion and parasite detection in the foetal brain in 83% of aborted foetuses (Arranz-Solis *et al.*, 2015b; Sánchez-Sánchez *et al.*, 2018), the challenge doses tested intravenously in the present study were less than the 10^6 tachyzoites previously assayed; tachyzoites were diluted 1:10 to a minimum concentration of 10^2 tachyzoites, similar to those evaluated by (Weston *et al.*, 2009). Subcutaneous inoculation closely mimics a natural primary infection as the parasite is "processed" through a draining lymph node before circulating in the blood (Dubey *et al.*, 2006). In cattle, it was reported that subcutaneous infection resulted in a foetal mortality that was 50% reduced compared to intravenous infection (Macaldowie *et al.*, 2004). Hence, in this study we investigated the outcome of *N. caninum* infection after subcutaneous inoculation in pregnant ewes using one of the intravenously tested doses. The dose of 10^4 tachyzoites was chosen for the subcutaneous administration since it is an intermediate-to-high dose between those tested by the intravenous route and also, because in a previous study, intravenous inoculation of $5x10^3$ tachyzoites of New Zealand isolates at mid-gestation resulted in abortion in 50% of the ewes (Weston *et al.*, 2009).

In this study, all pregnant ewes intravenously infected with 10^5 tachyzoites aborted in the same way as pregnant ewes intravenously infected with a 10-fold higher dose (10^6 tachyzoites) (Arranz-Solis *et al.*, 2015b; Sánchez-Sánchez *et al.*, 2018). The intravenous dose causing abortion in 50% of the infected animals was 10^2 tachyzoites. It is remarkably lower than the intravenous dose of $5x10^3$ causing abortion in 50% of the infected animals in the dose-titration study at mid-gestation using Nc-NZ1, Nc-NZ2 and Nc-NZ3 isolates (Weston *et al.*, 2009), suggesting higher virulence of Nc-Spain7 than New Zealand isolates in pregnant sheep. Likewise, intravenous infection at mid-gestation with 10^5 tachyzoites of the Nc-Spain7 isolate resulted in abortion of all pregnant ewes, whereas intravenous infection of pregnant ewes at mid-gestation with $1.7x10^5$ tachyzoites of a mixture of the Nc-2 and Nc-Liverpool isolates caused abortion in 67% of the pregnant ewes (McAllister *et al.*, 1996b). However, because (McAllister *et al.*, 1996b) and (Weston *et al.*, 2009) used a mixture of different isolates within the same inoculum to assure infection, it is difficult to establish comparisons with these studies (Benavides *et al.*, 2014). The median number of abortion days in our study was similar to the time range of abortions in previous *N. caninum* experimental infections in pregnant sheep at mid-gestation (McAllister *et al.*, 1996b; Buxton *et al.*, 1998; Buxton *et al.*, 2001; Arranz-Solis *et al.*, 2015b). As reported by Weston *et al.* (2009) regarding the differences in the average time between abortion and parturition found after infection with decreasing doses, in the present study, the median survival times in ewes infected intravenously with 10^3 tachyzoites and more markedly in ewes

intravenously infected with 10^2 tachyzoites were prolonged compared to those infected intravenously with 10^5 tachyzoites and 10^4 tachyzoites and to those subcutaneously challenged with 10^4 tachyzoites. In all aborting dams from groups infected intravenously with 10^3 and 10^2 tachyzoites, the coexistence at the time of euthanasia of live foetuses and dead foetuses in twin pregnancies suggests lower foetal damage in these groups because this observation was only found in one aborting dam in the group infected with 10^5 tachyzoites and one aborting dam in the group subcutaneously infected with 10^4 tachyzoites. Mummified foetuses found in group intravenously challenged with 10^5 and 10^4 tachyzoites have already been described after *N. caninum* experimental infection at mid-gestation in pregnant sheep (Buxton *et al.*, 1997). In groups intravenously infected with 10^3 and 10^2 tachyzoites and in the group subcutaneously challenged with 10^4 tachyzoites, pregnant ewes gave birth prematurely, similar to some pregnant ewes infected with $5x10^3$ *N. caninum* tachyzoites (Weston *et al.*, 2009). Consequently, in groups intravenously infected with 10^3 and 10^2 tachyzoites and in the group subcutaneously challenged with 10^4 tachyzoites, stillborns and lambs that died soon after birth show a significant decrease in their bodyweight, as previously described Buxton *et al.* (1998). Likewise, a more significant decrease in lamb weight in the group intravenously challenged with 10^3 tachyzoites was found because it is known that with increasing litter size, the weight of the lambs is lower (Gardner *et al.*, 2007). The presence of a large number of stillborn lambs and weak lambs dying within 24 h after birth could be explained by the absence of differences in parasite detection, parasite load and lesion severity from the foetal brain between aborted foetuses and lambs that gave birth in each group.

Traditionally, different temperature responses have been associated with the dose of parasite inoculums (Buxton *et al.*, 1997; Maley *et al.*, 2001; Maley *et al.*, 2003; Weston *et al.*, 2009). Intravenously challenged groups show a unique fever peak after infection, although a dose-dependent delay in the time of rectal temperature increase was found compared to the infection with 10^6 tachyzoites of the Nc-Spain7 isolate (Sánchez-Sánchez *et al.*, 2018), suggesting delayed parasite replication as lower infection doses were applied. However, as reported with 10^6 tachyzoites of the Nc-Spain7 isolate (Sánchez-Sánchez *et al.*, 2018), the groups intravenously infected with 10^5 and 10^4 tachyzoites show maximum rectal temperature on day 7 pi. Whereas the increase in rectal temperature persisted for 4 days in the groups intravenously infected with 10^5, 10^4 and 10^3 tachyzoites a less prolonged period with 3 days of rectal temperature increase was found in the group intravenously challenged with 10^2 tachyzoites. Similar to different temperature responses found between intravenous and subcutaneous *N. caninum* challenge in cattle (Macaldowie *et al.*, 2004), after subcutaneous challenge with 10^4 tachyzoites, a lower rectal temperature increase was found compared to intravenous challenge with the same dose. Likewise, a similar temperature response was found in group subcutaneously challenged with 10^4 tachyzoites compared to the infection in sheep with the same dose (10^4 tachyzoites) of the Nc-Liverpool isolate (Buxton *et al.*, 1997). The biphasic temperature response found in the subcutaneous challenge group had been previously described after subcutaneous challenge in sheep (Buxton *et al.*, 1997; Buxton *et al.*, 1998; Buxton *et al.*, 2001) and cattle (Maley *et al.*, 2003; Benavides *et al.*, 2012). Previous studies in cattle have described differences in rectal temperatures between aborting and non-aborting dams (Almería *et al.*, 2010; Almería *et al.*, 2016) in the same way as observed in the group intravenously challenged with 10^2 tachyzoites in the present study. The clinical evaluation in the group subcutaneously challenged with 10^4 tachyzoites revealed enlargement of the left prefemoral lymph node as previously described Maley *et al.* (2001), Maley *et al.* (2003), Macaldowie *et al.* (2004) and Rocchi *et al.* (2011) after subcutaneous *N. caninum* challenge in cattle.

Regarding cellular immune response, similar IFNγ kinetics, with very short lived IFNγ levels, have been described in previous reports carried out in cattle upon stimulation of peripheral blood mononuclear cells (PBMCs) (Regidor-Cerrillo *et al.*, 2014) or in sheep serum (Arranz-Solís *et al.*, 2016). In the group intravenously challenged with 10^5 tachyzoites, IFNγ released upon stimulation increased on day 7 pi in the same way that intravenous infection with 10^6 tachyzoites of the Nc-Spain7 isolate (Sánchez-Sánchez *et al.*, 2018). Nevertheless, the time course of IFNγ shows a delay until day 10 pi for IFNγ release after intravenous challenge in the groups intravenously challenged with 10^4 and 10^3 tachyzoites and in the group subcutaneously challenge with 10^4 tachyzoites. Likewise, no significant increase in IFNγ was observed in the group intravenously challenged with 10^2 tachyzoites which might have led to lower initial control of parasitaemia at the peripheral level, allowing a higher number of parasites to reach the placenta (Entrican, 2002; Innes, 2007). In fact, no differences in parasite load in the foetal brain between the groups intravenously challenged with 10^2 and 10^5 tachyzoites could be due to the absence of a significant increase in IFNγ levels in pregnant ewes from the group intravenously challenged with 10^2 tachyzoites because a threshold IFNγ response is required to be beneficial against *N. caninum* (Almeria *et al.*, 2014; Almería and López-Gatius, 2015). Recently, it has been shown that the immune response appears to lead to superior priming of a cell-mediated immune response in dams carrying live foetuses versus dams carrying dead foetuses (Bartley *et al.*, 2012; Darwich *et al.*, 2016). In this way, ewes that gave birth in the group intravenously challenged with 10^4 tachyzoites had higher IFNγ levels on day 10 pi than those that aborted. Although no significant differences were found in IFNγ levels in the group intravenously challenged with 10^2 tachyzoites, a delay in the IFNγ peak was detected in ewes that gave birth. That, along with differences in rectal temperatures between aborting ewes and ewes that gave birth could suggest decreased early-stage replication of the parasite in ewes that gave birth in the group intravenously challenged with 10^2 tachyzoites.

After intravenous infection of pregnant sheep at mid-gestation with 10^6 tachyzoites of the Nc-Spain7 isolate, IgG levels increased from day 12–14 pi (Arranz-Solís *et al.*, 2016; Sánchez-Sánchez *et al.*, 2018), whereas IgG levels increased from day 21 pi in the group intravenously challenged with 10^5 tachyzoites. Furthermore, the group intravenously infected with 10^5 tachyzoites exhibited higher IgG levels than those found with lower doses, possibly due to exposure to more abundant antigen and increased lymphoid stimulation similar to that reported by others (Buxton *et al.*, 1997; Maley *et al.*, 2001; Bartley *et al.*, 2004; Weston *et al.*, 2009). In this study, all challenged animals show seroconversion by ELISA, whereas the lower dose tested (50 tachyzoites of Nc-NZ1, Nc-NZ2 and Nc-NZ3 isolates) by (Weston *et al.*, 2009) revealed one seronegative animal at parturition by IFAT. Concerning offspring, all aborted foetuses and lambs from the infected groups had seropositive IFAT titres with no significant differences between challenge doses or routes of administration. Altogether, this finding indicates that once infection is established, it cannot be cleared from the host, and vertical transmission of the parasite occurred in all infected animals. In contrast, in a previous dose-titration study, none of the lambs and only 3 out of 5 lambs born from pregnant ewes intravenously infected with 50 and $5x10^3$ *N. caninum* tachyzoites (Nc-NZ1, Nc-NZ2 and Nc-NZ3 isolates), respectively, were seropositive by IFAT (Weston *et al.*, 2009).

Because immune responses are not accurate enough to be used as indicators for disease or protection (Benavides *et al.*, 2014), parasite detection and quantification and histopathological assessment are essential. All placentomes from infected ewes were PCR positive, and no significant differences in parasite load or lesion severity were found, so immune responses were unsuccessful in preventing the colonization and multiplication of *N. caninum* in the placentomes of aborting ewes. No difference was found in parasite detection between placentomes from intravenously infected ewes

at mid-gestation with 10^6 tachyzoites of the Nc-Spain7 isolate (PCR-positive samples from 83 to 100%) (Arranz-Solis *et al.*, 2015b; Sánchez-Sánchez *et al.*, 2018) and placentomes from intravenously infected aborting ewes in this study. *N. caninum* DNA was also widely detected in cotyledons from ewes that gave birth with no significant differences in parasite detection, similar to that reported by (Weston *et al.*, 2009), which found all PCR-positive cotyledons in ewes infected with $5x10^3$ *N. caninum* tachyzoites. However, lower parasite loads were found in cotyledons from ewes that gave birth in the group intravenously challenged with 10^3 tachyzoites, which, along with lower IFNγ levels detected, could suggest mild infection in these animals. When routes of administration were compared, no significant differences were identified in parasite detection, parasite load or lesion severity in placentomes, however, cotyledons from the subcutaneously infected group showed lower parasite burden compared to the corresponding group intravenously infected with a trend towards significance, maybe influenced by the lower number of animals in this group.

As for transplacental transmission, no significant differences were not found between doses among detection percentages in foetal brains from groups intravenously challenged with 10^5, 10^4 and 10^2 tachyzoites, nor when they were compared to detection in foetal brains after intravenous infection of pregnant ewes with 10^6 tachyzoites of the Nc-Spain7 isolate at mid-gestation (94% of PCR-positive samples) (Sánchez-Sánchez *et al.*, 2018). These results were not in accordance with differences in the proportion of positive brains in foetuses/lambs of a dose-titration study in pregnant sheep at mid-gestation using other isolates (Weston *et al.*, 2009). Conversely, the lower detection percentage in foetal brains from the group intravenously challenged with 10^3 tachyzoites could be because of the higher number of foetuses per dam in this group; fewer parasites that cross the placental barrier reach each foetus. Brain-negative foetuses arising from multiple pregnancies have already been reported in ewes infected at mid-gestation with 10^6 tachyzoites of Nc-Spain7 (Arranz-Solis *et al.*, 2015b). Foetuses and lambs showing PCR-negative brains in this study were seropositive by IFAT in the same way as in (Porto *et al.*, 2016), and brain lesions were identified, suggesting the presence of very low parasite load in their brains.

In brief, after intravenous infection with 10^5 tachyzoites foetal death is induced in all infected animals in the same way as 10^6 tachyzoites previously assayed. In addition, intravenous infection with 10^5 tachyzoites shows distinct immune responses and parasite load in the foetal brain. Surprisingly, the differences between the highest and the lowest intravenous doses were much smaller than expected, and we here demonstrate that experimental infection with as few as 100 tachyzoites could induce abortion in 50% of the ewes, and parasite load in the foetal brain was similar to that with the highest dose. Regarding the routes of inoculation, subcutaneous infection with 10^4 tachyzoites shows similar abortion rates and vertical transmission to intravenous infection. In light of these results, we propose that future studies using an abortion model for ovine neosporosis should be carried out using the intravenous route of administration and a challenge dose of 10^5 tachyzoites (100% abortion and vertical transmission), which will then allow to obtain more accurate and realistic conclusions in studies testing vaccine and drug candidates. However, further studies would be desirable to evaluate the outcome of infection with 10^5 tachyzoites by the subcutaneous route of administration.

Evaluation of safety and efficacy of BKI compounds in pregnant sheep experimentally infected with *T. gondii* and *N. caninum*

At present, there is lack of effective drugs for the treatment of congenital toxoplasmosis and neosporosis in ruminants (Sánchez-Sánchez et al., accepted). BKI compounds have emerged as promising drugs with tested efficacy against *T. gondii* and *N. caninum in vitro* and in laboratory animal models and also against other apicomplexan parasites (Van Voorhis *et al.*, 2017). However, their safety in pregnant ruminants and their efficacy in pregnant ruminant models of toxoplasmosis and neosporosis have not been assessed. For this reason, the third objective of the present Doctoral Thesis was to test the safety in pregnant sheep of BKI-1294 and BKI-1553 and their efficacy against abortion and vertical transmission after experimental infection with *T. gondii* and *N. caninum*, respectively. For the achievement of the third objective, pregnant ewes were orally dosed with BKI-1294 (5 doses of 100 mg/kg every other day) or subcutaneously dosed with BKI-1553 (with two different dosages: 1st dose of 35 mg/kg and a week later and a 2nd dose at 10 mg/kg or 7 doses at 10 mg/kg every other day). The efficacy of these drugs administered 48 hours after infection was evaluated in pregnant ewes infected orally with TgShSp1 oocysts (BKI-1294) or intravenously with Nc-Spain7 tachyzoites (BKI-1553). Safety of BKI compounds was assessed by clinical monitoring of rectal temperatures and foetal viability, evaluation of haematological and biochemical parameters and monitoring of fecal consistency (after oral administration) and local side effects (after subcutaneous administration). The efficacy was assessed with respect to the clinical course of disease, immune responses, lesion development and parasite presence in placental and/or target foetal tissues. In addition, drug levels of BKI-1294 and BKI-1553 in sheep plasma samples and in the fetal plasma samples after single administration of BKI-1553 at 10 mg/kg were determined.

In mice, BKI-1553 showed concentrations around 13 μM after oral administration of 10 mg/kg (Schaefer *et al.*, 2016; Vidadala *et al.*, 2016), however, BKI-1294 showed only levels of 0.75 μM using the same dose (Schaefer *et al.*, 2016; Hulverson *et al.*, 2017). In calves, BKI-1553 resulted in successful systemic exposure after a single oral dose of 10 mg/kg, with maximum concentrations of 10 μM (Schaefer *et al.*, 2016; Vidadala *et al.*, 2016), however, BKI-1294 resulted in plasma levels of 1 μM using the same dose (Schaefer *et al.*, 2016). In pregnant sheep, after subcutaneous administration of BKI-1553 at 10 and 35 mg/kg, maximum concentrations of >5 μM and trough concentrations of approximately 4 μM were found, however, oral administration of 100 mg/kg of BKI-1294 showed maximum concentrations of 2 μM, with trough plasma concentrations of 0.4-1 μM. Therefore, BKI-1553 has shown greatly improved bioavailability compared to BKI-1294 in different species. In addition, because toxoplasmosis and neosporosis greatly affects the foetus, foetal blood levels of BKI-1553 was evaluated in pregnant ewes. BKI-1553 application resulted in foetal plasma concentrations of 1.6 ± 0.2 μM, which corresponded to approximately 20–30% of the systemic exposure in pregnant ewes over 24-30 h post dosing. Previous studies addressing the *in vitro* efficacy of BKI-1553 against *N. caninum*, a half-maximal inhibitory concentration (IC_{50}) of 0.18 ± 0.03 μM was reported (Müller *et al.*, 2017b). Therefore, plasma concentrations of BKI-1553 in pregnant ewes and their foetuses were higher than the IC_{50} for *N. caninum*, perhaps indicating adequate exposure that could translate into good efficacy in the pregnant sheep model of neosporosis, since *in vitro* efficacy against *T. gondii* has not been assessed. Regarding the *in vitro* studies of BKI-1294 against the reference type II *T. gondii* isolate (TgME49) and the Nc-Spain7 isolate reported a half-maximal inhibitory concentration (IC_{50}) of 0.22 ± 0.06 μM and 0.27 ± 0.02, respectively (Winzer *et al.*, 2015). Thus, plasma concentrations of BKI-1294 in pregnant ewes were higher than the IC_{50} for *T. gondii* and *N. caninum*, perhaps indicating adequate exposure that could translate into good efficacy in the pregnant sheep models of toxoplasmosis and neosporosis.

Regarding safety, BKI-1553 at 100 mg/kg twice daily for 5 days in mice did not cause any adverse side effects leading to toxicity, and complete blood counts and serum biochemical profiles were within normal ranges, suggesting that the compound was safe. However, although no gross abnormalities upon necropsy were found, histological examination revealed inflammation in the spleen and liver in several treated mice (Vidadala *et al.*, 2016). Similarly, BKI-1294 at 100 mg/kg twice daily for 5 days in mice did not show any signs of toxicity or weight loss, and no alterations in tissue histology, metabolic enzymes, and complete blood counts were found (Ojo *et al.*, 2013). Concerning safety during pregnancy, when BKI-1553 was applied to pregnant mice daily at 20 mg/kg bodyweight for 5 days, it caused high neonatal mortality; this did not occur after administration at 20 mg/kg bodyweight 3 times with one-day intervals after each drug treatment (Müller *et al.*, 2017b). When BKI-1294 at 50 mg/kg for 5 days (Müller *et al.*, 2017c) was applied, decrease by half on mice fertility was found (Müller *et al.*, 2017c), however, no detrimental effect on fertility was found using BALB/c mice (Winzer *et al.*, 2015). In calves, BKI-1553 and BKI-1294 did not show toxicity after oral administration of 10 mg/kg (Schaefer *et al.*, 2016; Vidadala *et al.*, 2016). In pregnant ewes treated subcutaneously with BKI-1553, dermal nodules were found 24 h after drug application at the sites of administration in all of them. Similar dermal nodules were found after vehicle (70% Tween 80 and 30% Ethanol 96°) administration in sheep, indicating that the dermal nodules could be formed due to the vehicle. A formulation comprised of 7% Tween 80, 3% ethanol 96° and 90% normal saline has been already used in mice for oral administration of BKI-1294 (Ojo *et al.*, 2013) and in rats for oral and intravenous administration of antimalarial drugs (Van Voorhis *et al.*, 2007). In this study, higher percentage of Tween 80 and ethanol 96° was chosen as drug vehicle to achieve drug concentration that would allow the volume be administered subcutaneously. The much higher percentages of Tween 80 and Ethanol 96° could have triggered the appearance of mild dermal nodules. In pregnant ewes treated orally with BKI-1294 no alterations on the faecal consistency was found. Therefore, 5 doses orally of BKI-1294 at 100 mg/kg (formulated in 60% PHOSAL® 53 MCT, 30% Polyethyleneglycol 400 and 10% Ethanol 96º) seems to be safe in pregnant ewes.

Concerning systemic side effects, BKI-1294 by the oral route did not show increase on rectal temperature. By contrast, non-infected pregnant ewes treated subcutaneously with BKI-1553 exhibited an increase in rectal temperatures on day 1-2 posttreatment. Ewes treated with BKI-1294 and BKI-1553 did not show alterations on the hematological parameters, except a minor monocytosis found in group infected and treated subcutaneously with 7 doses of BKI-1553, which could have been derived from dermal nodules arising after 7 BKI-1553 administrations over short intervals. Additionally, it was suggested that monocyte levels increase during the recovery period during inflammation (Weiss and Perman, 1992). Biochemical parameters such as GGT, AST and ALT were consistently in the physiological range or irrelevantly increased in pregnant ewes treated with BKI-1294 and BKI-1553. The apparent lack of liver toxicity is important because liver metabolism is hypothesized to be predominant for BKI-1294 in mice (Ojo *et al.*, 2013) and might also occur for BKI-1553, since it is also based on the naphthalinyl-pyrazolopyrimidine scaffold. Despite only 1% of BKI-1294 being excreted in urine (Ojo *et al.*, 2013), renal function parameters are essential for toxicity evaluation. No remarkable abnormalities were found for urea and creatinine values after treatment with BKI-1294 and BKI-1553. CK showed levels above the physiological range in some treated groups with BKI-1294 and BKI-1553, although increased concentrations can typically appear in late pregnancy (Yokus *et al.*, 2006). CK and AST values have been previously described as valuable for investigating cardiotoxicity of drugs in lambs (Ekici and Isik, 2011). In contrast to BKI-1294, which was shown to be a potent human ether-ago-go-related gene (hERG) inhibitor, BKI-1553 does not have a hERG liability (Vidadala *et al.*, 2016). Myocardial damage in sheep treated with BKI-1294 and BKI-1553 was not identified, since the corresponding markers AST and CK were not

altered. No notable disorders related to minerals were observed after BKI-1294 and BKI-1553 administration. In spite of the decrease on fertility in mice by BKI-1294 and BKI-1553, no abortions were found in pregnant sheep treated with BKI-1294 and BKI-1553 and all lambs born healthy with no decrease on birthweight.

Regarding efficacy of BKI-1294 against *T. gondii* infection in pregnant sheep, although previous studies testing drugs orally administered started 10 days before the infection until parturition, (allowing better chance to control *T. gondii* infection), in our study with a shorter time window, treatment with 5 doses of BKI-1294 initiated 48 hours after infection resulted in higher protection against perinatal mortality (76%) compared to studies using monensin (46% of increase in live lambs) (Buxton *et al.*, 1988) and decoquinate (61% of protection against acute abortions) (Buxton *et al.*, 1996). Likewise, contrary to this study in which 100% perinatal mortality was found in infected but untreated pregnant ewes, only 53-55% of perinatal mortality was detected in infected but untreated pregnant ewes from studies testing monensin and decoquinate (Buxton *et al.*, 1988; Buxton *et al.*, 1996), indicating not so aggressive infection. In previous studies, a decreased birthweight has been described in lambs born form *T. gondii* infected ewes (Buxton *et al.*, 1996). In our study, lambs born from twin pregnancies in group infected and treated with BKI-1294 exhibited slightly lower birthweights compared to uninfected group receiving vehicle alone, but since no significant differences were found between lambs PCR-positive and PCR-negative in the brain, is likely that this lower growth of the foetuses could be consequence of a severely decrease on the food intake in the dams from infected and treated group for 6 days (from 96 to 101 days of pregnancy, associated to marked fever peak due to *T. gondii* infection) as previously described Gardner *et al.* (2007). Likewise, all lambs born healthy while in previous studies presented here using TgShSp1 in pregnant sheep, weak lambs were often observed. The rectal temperatures from *T. gondii* infected and BKI-1294 treated pregnant ewes were lower than infected but untreated pregnant ewes on days 5 and 7 pi, suggesting that the drug had an impact on parasite replication. Likewise, one day of delay on the onset of increase on rectal temperature was found in infected and treated ewes compared to those infected but untreated. Decreased rectal temperatures and/or delay in on the onset of rectal temperatures increase have also been described in experiments testing monensin (Buxton *et al.*, 1988) or decoquinate (Buxton *et al.*, 1996) against toxoplasmosis in pregnant ewes.

IFNγ is known to be important in inhibiting the intracellular multiplication of *T. gondii* and in addition will create the appropriate cytokine microenvironment for the priming of the adaptive immune response towards a Th-1 type pro-inflammatory immune response (Innes and Vermeulen, 2006). Analysis of the peripheral immune responses in pregnant ewes at different time points demonstrated a significant increase on the IFNγ release in stimulated peripheral blood cultures from infected and treated group on day 7 pi compared to infected but not treated group and uninfected group receiving vehicle alone. Likewise, infected and treated group showed a peak of IFNγ release on day 10 pi as well as 100-fold increased levels until delivery. *In vitro* studies showed that BKI-1294 induced the formation of intracellular multinucleated complexes composed of multiple pre-zoites unable to separate and form tachyzoites, but remaining viable for extended periods of time. These multinucleated complexes exhibit increased SAG1 expression, and also increased expression of the bradyzoite marker BAG1, with an overall heavily distorted parasite ultrastructure (Winzer *et al.*, 2015). If such complexes are also formed *in vivo*, they are unlikely to evade immune responses, but would be increasingly exposed to antigen-presenting cells, which would then result in higher IFNγ levels in treated animals. The SAG1 molecule is an immunodominant surface protein found on tachyzoites and is one of the most extensively studied antigens as it is able of inducing a T cell response with parasiticidal activity for extracellular *T. gondii* tachyzoites (Khan *et al.*, 1988).

Therefore, and similarly to the enhanced to potency of drugs against *T. gondii* observed *in vitro* (Radke *et al.*, 2018) and *in vivo* (Araujo and Remington, 1991) in the presence of IFNγ, the effect of BKI-1294 on *T. gondii* could act synergistically with IFNγ response. The increased levels of IFNγ in infected and treated group might have led to greater initial control of parasitaemia at the peripheral level, diminishing the numbers of parasites reaching and invading the placenta (Entrican, 2002). Concerning humoral immune responses, all animals infected with *T. gondii* and treated with BKI-1294 were seropositive using SAG1 antigen except one ewe that aborted on day 17 pi. However, by the ELISA based on *T. gondii* soluble antigens only one ewe that gave birth a stillborn lamb and two dams that gave healthy lambs seroconverted, while the remaining ewes were seronegative at the end of the sampling period. Since soluble antigens are exposed to the immune system during replication (Joiner and Roos, 2002), and since much lower humoral immune response to soluble antigens was found compared to ewes infected with lower oocyst doses (Sánchez-Sánchez, et al., submitted), it suggests low replication of the parasite throughout the experiment. Presence of SAG1 antibodies maybe derived from SAG1 expression in multinucleated complexes found *in vitro* after BKI-1294 treatment of *T. gondii* infected cultures (Winzer *et al.*, 2015). Likewise, it is known that the SAG1 antigen triggers an antibody response with an inhibitory effect on invasion (Mineo *et al.*, 1993).

In previous studies testing drugs against congenital toxoplasmosis in pregnant sheep, vertical transmission was evaluated through foetal serology and microscopic observation of lesions in placental tissues and foetal brains, with around 50% less of animals with vertical transmission after treatment with monensin (Buxton *et al.*, 1988), or only by evaluating placental tissues with 50% less of placentas showing lesions using decoquinate (Buxton *et al.*, 1996), albeit around 12% of infected and untreated sheep did not show placental lesions (Buxton *et al.*, 1988; Buxton *et al.*, 1996)or seropositive offspring (Buxton *et al.*, 1988). However, the study evaluating sulphamezathine/pyrimethamine showed 100% vertical transmission in untreated animals and 50% reduction on the placentas with lesions, but not differences on foetal serology in treated ones (Buxton *et al.*, 1993a). In our study testing efficacy of BKI-1294 against *T. gondii* infection, we evaluated transplacental transmission of the parasite through foetal serology and parasite detection and histological lesions in foetal tissues. In all the lambs born after infection with a 100-fold lower TgShSp1 oocyst dose than used in BKI-1294 efficacy experiment, *T. gondii* was detected in all their brains and lungs and lesions was found in most of the brains as described in the objective 1 of the present Doctoral Thesis. However, in lungs from lambs born in BKI-1294 treated group, known as a predilection site for *T. gondii* (Gutierrez *et al.*, 2010), no significant lesions or parasites were detected. Likewise, in foetal brains, parasites were not detected in 7 lambs born healthy and slightly detected in the brain of 6 of the 13 lambs born healthy (53% of protection against vertical transmission in lambs born), while no lesions in the brain were found in any of them. Therefore, there was a slight dissemination of the parasite in the lambs with no antibodies detected by IFAT, although this technique could show low sensitivity (Castaño *et al.*, 2016). These results are consistent with those from a pregnant mouse model of toxoplasmosis, in which 100% protection against pup mortality and 93% of protection against vertical transmission in the surviving offspring were accomplished with BKI-1294 (Müller *et al.*, 2017). The higher protection of BKI-1294 in pregnant mice compared to pregnant sheep could be explained by the lower perinatal mortality and vertical transmission of *T. gondii* type II isolates in mice compared to sheep as described in the objective 1 of the present Doctoral Thesis. Since BKI-1294 triggers formation of multinucleated complexes in *T. gondii* infected cultures (Winzer *et al.*, 2015), maybe *in vivo*, is likely that some tachyzoites trapped within the host cell could restart their replication and therefore induce perinatal mortality or vertical transmission. In fact, in some ewes the parasites reach the foetus, but since is likely that therapeutic concentrations of BKI-1294 might have been reached in the foetus, similarly to the results described

above for BKI-1553, the dissemination of the parasite could be controlled. However, it could happens that the central nervous system penetration of the drug did not allow drug levels above IC_{50} in the foetal brain, which resulted in replication of the parasite in some cases.

As for efficacy of BKI-1553 against *N. caninum* infection in pregnant sheep, clinical observations in the BKI-1553 treated groups revealed a partial protection against abortion, as 37 and 50 percent of the pregnant ewes gave birth in group infected and treated twice and group infected and treated seven times, respectively, with statistically improved foetal survival rates and median foetal survival times in the group infected and treated seven times. However, lambs in group infected but treated seven times with BKI-1553 exhibited lower birthweights compared to uninfected and untreated lambs, therefore since no decrease in birthweights was found in the corresponding treated group but uninfected, the infection, but not the treatment, impacted on the weight in this group. The rectal temperatures from pregnant ewes in the group infected and treated twice with BKI-1553 were lower than uninfected and untreated group on days 5 and 6 pi, suggesting that the drug had an impact on parasite replication. A decrease in rectal temperatures has also been described in experiments testing toltrazuril against neosporosis in calves (Kritzner *et al.*, 2002). Additionally, since AST typically increases after *T. gondii* infection, indicating liver injury (Yeo *et al.*, 2016), the increased AST in infected but untreated group and not in both groups infected and treated at the final time point might indicate that treated pregnant ewes exerted better control of *N. caninum* infection. IFNγ levels were higher on day 7 pi in both groups infected with *N. caninum* and treated with BKI-1553 and on day 14 pi in the group infected and treated seven times with BKI-1553, compared to group infected but not treated. Likewise, increased IFNγ levels in group infected and treated 7 times on day 14 pi might be caused by extended BKI-1553 administration in this group. The increased levels of IFNγ in both groups infected and treated with BKI-1553, similarly to animals infected with *T. gondii* and treated with BKI-1294 might have led to greater initial control of parasitaemia at the peripheral level, diminishing the numbers of parasites reaching and invading the placenta (Entrican, 2002; Innes, 2007). Concerning humoral immune responses, antibody responses in the treated groups were delayed. Also, on day 21 pi, significantly decreased IgG levels were found in both groups infected and treated with BKI-1553, but group infected and treated seven times with BKI-1553 showed lower IgG values than the other two infected groups. Similarly decreased antibody responses have been previously reported during treatments of *N. caninum* infected calves with toltrazuril (Kritzner *et al.*, 2002) and monensin (Buxton *et al.*, 1988) or decoquinate (Buxton *et al.*, 1996) treatment trials against toxoplasmosis in pregnant ewes. In treated groups, the delay at day 14 pi and lower antibody responses from day 21 pi onwards may have been due to higher IFNγ levels in the early stage of infection, as previously described Lopez-Gatius *et al.* (2007) under natural conditions. Likewise, longer IFNγ responses in the group infected and treated seven times with BKI-1553 could contribute to reduced IgG levels on day 21 in this group. In contrast, on day 21 pi, IgG levels from pregnant ewes that aborted in the group infected and treated twice with BKI-1553 were higher than those that gave birth, suggesting that higher antibody responses occurred in animals that aborted their foetuses, as previously described Almería *et al.* (2016).

While BKI-1553 treatment had a beneficial impact on offspring survival of *N. caninum* infected ewes, the drug did not prevent transplacental transmission. All foetuses/lambs were seropositive as assessed by IFAT. However, lower median IFAT titres were found in aborted foetuses from the treated groups, which is indicative for decreased antigen stimulation and thus enhanced control of *N. caninum* infection. To quantify the efficacy of BKI-1553 against transplacental transmission of N. caninum, microscopic lesions, detection and burden of *N. caninum* in placental tissues and foetal brains were investigated. In placental tissues *N. caninum* was widely detected in infected groups, as

well as lesions in all placentomes from aborting ewes. In foetal brains, known as a predilection site for *N. caninum* (Collantes-Fernández *et al.*, 2006), parasites were less abundant, and the percentage of foetal brains with histological lesions was reduced in the treated groups. This is likely due to the efficacious BKI-1553 concentrations present in the foetuses. These results are consistent with those from a pregnant mouse model of neosporosis, in which reduced transplacental transmission to offspring was accomplished (Müller *et al.*, 2017b). In the treated groups, histopathological analysis revealed a lower percentage of foetal brains with lesions, lower numbers of foci, and lower percentage of damaged areas in lambs that were born compared to aborted foetuses, which is in accordance to previously described studies (Macaldowie *et al.*, 2004). Additionally, the parasite burden in foetal brains was reduced in the group infected and treated seven times with BKI-1553, but not in the group infected and treated twice. However, in aborted foetuses in the group infected and treated twice, but not in the group infected and treated seven times, a higher parasite load in the brain was found compared to lambs born. Thus, the treatment with BKI-1553 seven times appeared to show higher efficacy in terms of controlling brain infection in the offspring. In both infected and treated groups, cerebral parasite loads in lambs born were found to be lower compared to cerebral parasite loads in aborted foetuses in the group infected but not treated. Central nervous system (CNS) penetration by BKI-1553 could possibly explain why, despite a lack of difference in systemic exposures, some foetuses aborted and others were protected. CNS penetration of BKI-1553 was previously found to be approximately 33% compared to plasma exposure in mice (Vidadala *et al.*, 2016). If this is similar in sheep, then BKI-1553 concentrations in foetal brains would be ≤2 fold above the *N. canimum* IC_{50} level at the troughs for all dose concentrations. Such rather low concentrations would most likely only offer incomplete protection at this infection site in foetuses.

To sum up, BKI-1294 and BKI-1553 reduced in 37-76% the foetal mortality after experimental infections with *T. gondii* and *N. caninum*, in which all the untreated animals aborted. Likewise, in dams, both BKI compounds decreased rectal temperature upon infection and triggered an increase in peripheral IFNγ levels. In the offspring, BKI-1294 prevented vertical transmission in 53% of lambs born from ewes infected with *T. gondii*, while BKI-1553 treatment did not prevent vertical transmission, but partially alleviated the effects of *N. caninum* infection, by reducing lesions, parasite presence and parasite loads in foetal brains. Looking ahead, other members of the BKI class of compounds under development with improved biovailability in mice and hERG liability, but also highly efficacious *in vitro* and in non-pregnant and pregnant mice models could be tested in the near future against toxoplasmosis and neosporosis in ruminants. In addition, since pregnant sheep model of toxoplasmosis may closely mimics the human infection with *T. gondii*, as explained above, the results obtained in sheep with this BKI compounds could be extrapolable to humans.

CAPÍTULO VI

CONCLUSIONES/CONCLUSIONS

Objetivo 1. Normalización de los modelos murino y ovino gestantes de infección por *T. gondii* y comparación de la virulencia de aislados tipo II de *T. gondii* en ratones y ovejas

Primera. Se observa mayor crecimiento y formación de quistes *in vitro* con el aislado TgME49 y mayor mortalidad de las crías y transmisión vertical tras la infección de ratones gestantes con el aislado TgME49 en comparación con el aislado recientemente obtenido TgShSp1. Estos resultados sugieren un incremento en la virulencia del aislado TgME49, probablemente debido a los pases sucesivos en cultivo celular y en ratones. Por lo tanto, se recomienda el uso de pases bajos en futuros estudios que comparen características fenotípicas entre aislados de *T. gondii*.

Segunda. La baja morbilidad y la ausencia de mortalidad en ratones no gestantes ni en gestantes inoculados por vía intraperitoneal con taquizoitos o por vía oral con ooquistes del aislado TgShSp1 sugieren una baja patogenicidad de este aislado para ratones adultos.

Tercera. En ovejas desafiadas a mitad de gestación con ooquistes no se encuentran diferencias en la mortalidad perinatal ni en las lesiones ni en el número de corderos positivos a *T. gondii* entre los aislados TgME49 y TgShSp1.

Cuarta. La inoculación de ratones y ovejas a mitad de la gestación con dosis similares de ooquistes del aislado TgShSp1 ocasionan una mayor mortalidad perinatal en ovejas que en ratones. Todas crias de ratones que sobreviven tras la infección durante la gestación con 100 ooquistes del aislado TgShSp1 fueron positivas a *T. gondii* al igual que todos los corderos mortinatos y nacidos vivos de ovejas desafiadas con 50 ooquistes del aislado TgShSp1. Sin embargo, mientras que todos los corderos mortinatos y nacidos vivos de ovejas desafiadas con 10 ooquistes del aislado TgShSp1 fueron positivos, tan solo el 37% de las crias de ratones que sobreviven a la infección durante la gestación con 25 ooquistes del aislado TgShSp1 fueron positivas a *T. gondii*. Por lo tanto, la virulencia en ratones con aislados de tipo II no es un buen indicador para predecir las consecuencias de la infección en ovejas gestantes.

Objetivo 2. Normalización de un modelo ovino gestante de infección con *N. caninum* mediante una titulación de dosis y evaluación de diferentes vías de administración del aislado virulento Nc-Spain7

Primera. Tan solo las ovejas desafíadas con la mayor dosis por vía intravenosa testada (10^5 taquizoitos) muestran 100% de aborto. Estas ovejas presentan un incremento más temprano de la temperatura rectal y de los niveles de IFN-gamma y una mayor respuesta inmune humoral. Además, su carga parasitaria en el cerebro fetal en comparación con aquellas ovejas desafiadas por vía intravenosa con dosis menores es también mayor.

Segunda. Las ovejas desafiadas por vía subcutánea con 10^4 taquizoitos muestran menor temperatura rectal y niveles de IFN-gamma en comparación con aquellas ovejas desafiadas por vía intravenosa con la misma dosis de taquizoitos, sin embargo no se observan diferencias entre ambas vías de administración en la respuesta inmune humoral ni la mortalidad fetal ni en la transmisión vertical.

Tercera. Se propone un modelo ovino gestante de primoinfección con *N. caninum* basado en la administración intravenosa de 10^5 taquizoitos. Esta dosis, 10 veces menor que la estudiada anteriormente, y esta vía de administración parecen ser suficientes para provocar el 100% de aborto. Esta modificación permitirá obtener resultados más precisos en ensayos de candidatos terapeúticos y vacunales.

Objetivo 3. Evaluación de la seguridad y eficacia frente a *T. gondii* y *N. caninum* de los compuestos BKI en ovejas gestantes.

Primera. La administración oral del BKI-1294 y la administración subcutánea del BKI-1553 en ovejas gestantes a las pautas de dosificación estudiadas da lugar a concentraciones plasmáticas terapéuticas para *T. gondii* y *N. caninum* respectivamente. Sin embargo, el BKI-1553 y de forma más acusada el BKI-1294 presentan un aclaramiento plasmático rápido. El BKI-1553 parece tener una mejor farmacocinética que el BKI-1294 en ovejas gestantes, de forma similar a como se describió previamente en ratones y terneros. Además el BKI-1553 es capaz de atravesar la barrera placentaria, encontrándose concentraciones terapéuticas de este fármaco en los fetos.

Segunda. Tanto el BKI-1294 como el BKI-1553 parecen ser seguros ya que no se han encontrado alteraciones de la temperatura rectal ni de los parámetros hematológicos y bioquímicos ni de la gestación o daño local asociado a estos fármacos. Sin embargo, se ha observado diferente seguridad en función de la vía de administración. Mientras que la administración oral no incrementó la temperatura rectal ni modificó la consistencia fecal, la administración subcutánea provocó la aparición de nódulos dérmicos asociados a un incremento de temperatura rectal y a una monocitosis.

Tercera. La administración del BKI-1294 a ovejas infectadas con *T. gondii* durante la gestación se asocia a un menor incremento de las temperaturas rectales, a unos niveles mayores de IFN-gamma a nivel periférico que en ovejas no tratadas, a una menor respuesta inmunitaria humoral a antígenos solubles pero a niveles elevados de anticuerpos anti-SAG1 y una disminución de la mortalidad perinatal del 76%. En la descendencia, el BKI-1294 evita la transmisión vertical en el 53% de los corderos. Por tanto, la eficacia del BKI-1294 es mayor comparada con aquella observada previamente con monensina, decoquinato e inhibidores de la síntesis del folato.

Cuarta. La administración del BKI-1553 a ovejas infectadas con *N. caninum* durante de la gestación se asocia a un menor incremento de las temperaturas rectales, produce un incremento del IFN-gamma, reduce los niveles de IgG específicas a nivel periférico y consigue reducir in 37-50% la tasa de abortos. En los fetos, la administración del BKI-1553 no evita la transmisión vertical pero disminuye los efectos de la infección, con una reducción de las lesiones y de la presencia y carga del parásito en el cerebro fetal.

Objective 1. Standardization of pregnant mice and sheep models of *T. gondii* infection and comparison of the virulence of *T. gondii* type II isolates in mice and sheep.

First. Higher growth and *in vitro* cyst formation for the TgME49 isolate and higher pup mortality and vertical transmission are observed after infection of pregnant mice with the laboratory TgME49 isolate compared to the recently obtained TgShSp1 isolate. These results suggest an enhanced virulence for the TgME49 isolate, maybe due to successive passages in cell culture and mice. Therefore, it could be recommended the use of low passage stocks for future studies comparing phenotypic traits between *T. gondii* isolates.

Second. The low morbidity and absence of mortality in non-pregnant and pregnant mice inoculated either with tachyzoites intraperitoneally or with oocysts by the oral route using the TgShSp1 isolate suggest a low pathogenic effect for this isolate in adult mice.

Third. In sheep challenged at mid-pregnancy with oocysts no differences are present on the perinatal mortality or lesions and number of *T. gondii*-positive lambs between TgME49 and TgShSp1 isolates.

Fourth. Inoculation of mice and sheep at mid-pregnancy with similar TgShSp1 oocyst doses induce higher perinatal mortality rates in sheep than in mice. All surviving offspring from mice inoculated with 100 TgShSp1 oocysts were positive for *T. gondii* in the same way as stillbirths/lambs born alive from ewes infected with 50 TgShSp1 oocysts. However, while all stillbirths/lambs born alive from ewes challenged with 10 TgShSp1 oocysts were positive for *T. gondii*, only 37% of the surviving offspring from mice inoculated with 25 TgShSp1 oocysts were positive for *T. gondii*. Consequently, the virulence of *T. gondii* type II isolates in pregnant mice is not a reliable predictor for the outcome of infection in pregnant sheep.

Objective 2. Standardization of the pregnant sheep model of *N. caninum* infection by a dose-titration assay and evaluation of different routes of administration of the virulent Nc-Spain7 isolate.

First. Only ewes challenged with the higher intravenous dose used in this study (10^5 tachyzoites) show 100% abortion. This ewes exhibit an earlier increase on rectal temperature and IFN-gamma levels and higher humoral immune responses. In addition, parasite load in the foetal brain compared to ewes challenged intravenously with lower doses is also higher.

Second. Ewes challenged subcutaneously with 10^4 tachyzoites exhibited lower rectal temperature and IFN-gamma levels compared to ewes challenged intravenously with the same dose of tachyzoites. However, no differences between both routes of administration are found on humoral immune responses, foetal mortality and vertical transmission.

Third. A pregnant sheep model of primoinfection with *N. caninum* based on intravenous inoculation of 10^5 tachyzoites is proposed. This dose, 10 times lower than previously assayed, and infection route, seems to be sufficient to trigger 100% abortion. This adjustment will allow to obtain more accurate results in studies testing vaccine and drug candidates.

Objective 3. Evaluation of the safety and efficacy against *T. gondii* and *N. caninum* of BKI compounds in pregnant sheep.

First. The oral administration of BKI-1294 and the subcutaneous administration of BKI-1553 in pregnant sheep at the dose regimes assayed provide therapeutic plasmatic levels for *T. gondii* and *N. caninum*, respectively. However, BKI-1553 and more markedly BKI-1294 exhibit a rapid plasma clearance. BKI-1553 seems to have a better pharmacokinetic profile than BKI-1294 in pregnant sheep, in accordance to previous reports in mice and calves. Additionally, BKI-1553 is able to cross the placental barrier since therapeutic concentrations of this drug are found in the foetuses.

Second. Both, BKI-1294 and BKI-1553, seem to be safe since no alterations on rectal temperature, hematological and biochemical parameters, pregnancy or local damage are associated with the drugs. However, differences on safety between both routes of administration are found. While oral administration does not increase rectal temperature or modifies fecal consistency, subcutaneous administration induces the formation of dermal nodules with associated increase on rectal temperature and monocytosis.

Third. The administration of BKI-1294 to *T. gondii* infected ewes during pregnancy is associated with a lower rectal temperature increase, a higher peripheral IFN-gamma than untreated ewes, a low humoral immune response to soluble antigens but high levels of anti-SAG1 antibodies, and a decrease of 76% in the perinatal mortality. In the offspring, BKI-1294 prevents vertical transmission in 53% of the lambs. Therefore, the efficacy of BKI-1294 is higher than those observed previously using monensin, decoquinate and folate inhibitors.

Fourth. The administration of BKI-1553 to *N. caninum* infected ewes during pregnancy causes a lower rectal temperature increase upon infection, triggers an increase in peripheral IFN-gamma levels and a reduction in the specific IgG responses, and achieves a reduction of 37-50% in the abortion rate. In foetuses, vertical transmission is not prevented, but the effects of infection are partially alleviated by reducing lesions, parasite presence and parasite loads in foetal brains.

CAPÍTULO VII

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ANEXO I/APPENDIX I

Artículo de revisión

Tratamiento de la toxoplasmosis y neosporosis en rumiantes: conocimiento actual y perspectivas futuras

La toxoplasmosis y neosporosis son enfermedades causadas por protozoos ampliamente relacionadas entre si que ocasionan importantes pérdidas económicas en rumiantes de producción. La infección por *Toxoplasma gondii* causa principalmente problemas reproductivos en pequeños rumiantes y es una zoonosis ampliamente distribuida, mientras que la infección por *Neospora caninum* es una de las principales causas de aborto en ganado vacuno en todo el mundo. La vacunación ha sido considerada la medida de control más rentable economicamente para controlar estas enfermades. Sin embargo, a pesar de los esfuerzos en el desarrollo de vacunas, solo una vacuna viva atenuada de *T. gondii* ha sido registrada para su uso en medicina veterinaria., y no se han conseguido vacunas prometedoras frente a la neosporosis; por lo tanto el desarrollo de vacunas es un objetivo primordial. Adicionalmente, el tratamiento farmacológico podría ser una estrategia valiosa para el control de estas enfermedades en rumiantes de producción ya que varios fármacos que limitan la proliferación y diseminación han sido evaluados. Asimismo, experimentos en animales de producción podrían ser relavantes como testaje inicial de fármacos frente a parásitos zoonóticos. Los tratamientos pueden ser aplicados frente a infecciones en rumiantes adultos para minimizar las consecuencias de una primoinfeccion o de la reactivación de una infección crónica durante la gestación ó en rumiantes recién nacidos para evitar la cronificación de la infección. En esta revisión se presenta el conocimiento actual del desarrollo de fármacos frente a la toxoplasmosis y la neosporosis en rumiantes, y en un intento de impulsar otras opcionas fármacológicas adicionales, se recogen potenciales fármacos que han mostrado eficacia *in vitro* y en modelos de animales de laboratorio frente a la toxoplasmosis y neosporosis.

Treatment of toxoplasmosis and neosporosis in farm ruminants: state of knowledge and future trends

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Accepted for publication in *Current Topics in Medicinal Chemistry* (21th March 2018). It will be published in Volume 18. Reference "BMS-CTMC-2018-HT35-454-4"

Abstract

Toxoplasmosis and neosporosis are closely related protozoan diseases that lead to important economic impacts in farm ruminants. *Toxoplasma gondii* infection mainly causes reproductive failure in small ruminants and is a widespread zoonosis, whereas *Neospora caninum* infection is one of the most important causes of abortion in cattle worldwide. Vaccination has been considered the most economic measure for controlling these diseases. However, despite vaccine development efforts, only a live-attenuated *T. gondii* vaccine has been licensed for veterinary use, and no promising vaccines against neosporosis have been developed; therefore, vaccine development remains a key goal. Additionally, drug therapy could be a valuable strategy for disease control in farm ruminants, as several drugs that limit *T. gondii* and *N. caninum* proliferation and dissemination have been evaluated. This approach may also be relevant to performing an initial drug screening for potential human therapy for zoonotic parasites. Treatments can be applied against infections in adult ruminants to minimize the outcomes of a primo-infection or the reactivation of a chronic infection during gestation or in newborn ruminants to avoid infection chronification. In this review, the current status of drug development against toxoplasmosis and neosporosis in farm ruminants is presented, and in an effort to promote additional treatment options, prospective drugs that have shown efficacy *in vitro* and in laboratory animal models of toxoplasmosis and neosporosis are examined.

Keywords

Toxoplasma, *Neospora*, cattle, sheep, goats, chemotherapy, chemoprophylaxis.

Introduction

The cosmopolitan obligate intracellular protozoan parasites *Toxoplasma gondii* and *Neospora caninum* are considered major causes of reproductive failure in small ruminants and cattle, respectively (Dubey *et al.*, 2007; Dubey, 2009b; Reichel *et al.*, 2013). Thereby, toxoplasmosis and neosporosis deserve special attention since they lead to severe economic losses to ruminant livestock producers (Trees *et al.*, 1999; Innes *et al.*, 2009; Reichel *et al.*, 2013). In fact, the negative economic effects are more devastating in the case of epidemic abortion storms than in herds/flocks with endemic cases (Reichel *et al.*, 2013; Castaño *et al.*, 2016). In addition, despite the undoubtedly under-detection and underreporting of cases (Olivier *et al.*, 2007; Halsby *et al.*, 2014), toxoplasmosis affects 25-50% of the world population (Montoya and Liesenfeld, 2004; Flegr *et al.*, 2014; Neville *et al.*, 2015) and it is recognized as the parasitic zoonosis with the highest human incidence by the European Food Safety Authority (EFSA) (Olivier *et al.*, 2007).

T. gondii and *N. caninum* are closely related apicomplexan parasites, sharing many morphological and biological features and showing remarkably conserved genomes (Mugridge *et al.*, 1999; Speer *et al.*, 1999; Reid *et al.*, 2012). Moreover, they present similar facultative heteroxenous coccidian life cycles, including three invasive stages: i) the rapidly replicating and abortion-causing tachyzoites, ii) the slowly replicating or quiescent bradyzoites harboured in tissue cysts, especially those within the brain and skeletal muscle, and iii) the sporulated oocysts bearing sporozoites (Dubey *et al.*, 1998; Hemphill and Gottstein, 2006; Robert-Gangneux and Darde, 2012). However, large differences are found between them in relation to the host range of definitive and intermediate hosts, the zoonotic potential, which has been proven for only *T. gondii*, and the importance of horizontal and vertical routes of transmission in maintaining natural infections in the ruminant populations (Dubey and Schares, 2011; Robert-Gangneux and Darde, 2012).

Parasites and diseases

Host range and transmission

According to current knowledge, the members of the family Felidae, especially domestic cats (*Felis catus*), are the definitive hosts of *T. gondii*. They commonly become infected after eating wild rodents and birds containing *T. gondii* tissue cysts (bradyzoites) and, to a lesser extent, by ingesting oocysts shed by other cats (Dubey, 1993; Innes *et al.*, 2009). In turn, any warm-blooded mammal, including humans and birds, can act as an intermediate host (Innes *et al.*, 2009).

Most infections in sheep and goat flocks occur postnatally, mainly by the horizontal route after the ingestion of water and food contaminated with viable sporulated oocysts shed by cats (Dubey, 2009b; Innes *et al.*, 2009). However, the vertical transmission of *T. gondii* (from the dam to the foetus during gestation) can also occur in these two small ruminant species by both ‘exogenous transplacental transmission’ (ExTT) and ‘endogenous transplacental transmission’ (EnTT) mechanisms. The former mechanism, which is the most frequent, occurs during pregnancy as a consequence of an exogenous source while the latter, whose importance remains controversial (Innes *et al.*, 2009), occurs after the recrudescence of a latent infection acquired in utero by the reactivation of bradyzoites and their reconversion to tachyzoites. In human hosts, the consumption of raw or undercooked meat with *T. gondii* tissue cysts containing the bradyzoite stage is commonly considered the main source of infections in developed countries (Kijlstra and Jongert, 2009), although water is increasingly being investigated as a risk factor. In fact, oocyst contamination has been demonstrated to be an important source of infection in tropical and subtropical countries, and also in some European countries (Jones and Dubey, 2010; Hussain *et al.*, 2017).

The domestic dog (*Canis lupus familiaris*) and wild canids, such as American coyote (*Canis latrans*), Australian dingo (*Canis lupus dingo*) and Eurasian grey wolf (*Canis lupus lupus*) are the definitive hosts of *N. caninum*, while farm ruminants (cattle and sheep), horses and wildlife white-tailed deer and water buffalo are assumed to be intermediate hosts in the life cycle of *N. caninum* based on its successful isolation (Dubey and Schares, 2011).

Dogs are usually infected through consumption of *N. caninum*-infected bovine tissues (mainly foetuses and placentas). Cattle can become infected through the oral uptake of *N. caninum* sporulated oocysts that are shed by dogs from the farm environment (Hall *et al.*, 2005; McCann *et al.*, 2007), but the relative frequency with which cows are infected postnatally compared to that of cows infected by transplacental transmission remains unknown (Williams *et al.*, 2009). Highly efficient transplacental infections (ExTT and EnTT) through tachyzoites are responsible for the majority of natural neosporosis outbreaks in livestock (Davison *et al.*, 1999; Trees and Williams, 2005; Ortega-Mora *et al.*, 2006). As mentioned above for toxoplasmosis, ExTT of *N. caninum* is associated with dams that are oocyst-derived primo-infected during gestation, while EnTT of *N. caninum* is related to the reactivation and recrudescence of a previous latent infection during gestation. The efficiency of transplacental infection has been estimated to range from 44% (Bergeron *et al.*, 2000) to over 95% (Davison *et al.*, 1999), according to precolostral serology in calves born to seropositive dams. In fact, this route of infection is crucial to maintaining the infection within cattle herds, especially in herds with a high prevalence of seropositive cows (Bergeron *et al.*, 2000). Nevertheless, it has been proposed that transplacental transmission alone is not enough to maintain the infection within the herds, and thus horizontal transmission is required (French *et al.*, 1999; Bartels *et al.*, 2007).

Pathogenesis: outcome of infection in pregnant ruminants

The complex pathogenesis of both reproductive ruminant diseases is not fully understood (Dubey *et al.*, 2006; Dubey *et al.*, 2007), and there is still much to know concerning the role of parasite multiplication in the target tissues, maternal-foetal immunity (Buxton *et al.*, 2002) and other essential factors, such as variations in virulence and parasite stages. However, it is well documented in the literature that the outcome of toxoplasmosis, as well as the effects of neosporosis in pregnant sheep, goats and cattle, are strongly associated with the period of gestation at the time of primary infection (Buxton and Finlayson, 1986; Lindsay *et al.*, 1995; Arranz-Solis *et al.*, 2015b; Porto *et al.*, 2016).

Ewes infected prior to mating will develop an anti-*T. gondii* immune response, enhancing protection against abortion in subsequent pregnancies (Innes *et al.*, 2009), whereas repeated abortion in the next pregnancies has been described in goats (Dubey, 1982). In particular, if *T. gondii* tachyzoites reach a relatively immunologically immature foetus during the early stages of pregnancy, the infection usually results in foetal death. In turn, the birth of a stillborn or weak lamb, which is sometimes accompanied by a small, mummified foetus, is highlighted at mid-gestation stages, whereas the birth of healthy and clinically normal lambs that are congenitally infected is the main finding for ewes with late *T. gondii* infections or after the recrudescence of an endogenous infection (Trees and Williams, 2005).

Typically, *N. caninum* infection leads to a latent and asymptomatic course in non-pregnant cows. Conversely, bovine neosporosis in pregnant cows is associated with repeated abortion and the birth of clinically healthy, but persistently infected, calves (Buxton *et al.*, 2002). The likelihood of abortion by *N. caninum* also greatly depends on the period of

gestation in which the infection occurs. Although *N. caninum*-infected cows of any age may abort from 3 months gestation to term, most abortions are concentrated in the mid-gestation stages, between 5 to 7 months of gestation (Thurmond and Hietala, 1997a; Pereira-Bueno *et al.*, 2003; Collantes-Fernández *et al.*, 2006b). The outcome of infection at the late stages of gestation is consistent with most foetuses going-on-to-term, and thus these infections lead to the birth of congenitally infected calves that are clinically normal, but persistently infected (Pereira-Bueno *et al.*, 2000; Quintanilla-Gozalo *et al.*, 2000). A limited number of calves may show neuromuscular disorders (De *et al.*, 2005). It is also possible that congenitally infected cows may abort in successive gestations. In fact, around one in twenty cows that aborted once due to an *N. caninum* infection will abort again (Thurmond and Hietala, 1996; Ortega-Mora *et al.*, 2006).

Control

For the control of toxoplasmosis and neosporosis, different measures have been suggested; however, the combination of different approaches is known to be the optimal strategy (Dubey *et al.*, 2007; Dubey, 2009b). The implementation of farm biosecurity protocols, hygienic measures and management practices should be adopted in all farms, for reducing the level of environmental contamination with *T. gondii* oocysts via cat faeces or *N. caninum* oocysts via dog faeces (if present) and for avoiding novel infections through the introduction of infected animals to the herd. Among the biosafety practices that should be conducted to achieve this aim, the following are highlighted: limit cat and dog access to ruminant areas, especially to those housing pregnant ruminants, or to the areas for food storage and water supplies; promptly remove of placentas or foetal materials; appropriately dispose of dead livestock; and establish rodent control. Reproductive practices considering the artificial insemination of seropositive dams or the use of embryo transfer and test and cull strategies based on serological diagnosis are also recommended for the control of *N. caninum* infections (Dubey *et al.*, 2007; Reichel *et al.*, 2014; McAllister, 2016). Despite being properly designed and meticulously practised, globally, these control measures alone are not cost-viable or completely effective in eliminating toxoplasmosis and neosporosis from a herd, and it is necessary to complement them with an immune-chemotherapeutical approach (Zhang *et al.*, 2013; Hemphill *et al.*, 2016; McAllister, 2016).

In particular, vaccination has been considered a promising and economically viable solution to preventing the transmission of both infectious agents, despite its two current principal limitations related to efficacy and safety (Innes *et al.*, 2009; Horcajo *et al.*, 2016). In fact, to date, the control of ovine toxoplasmosis is primarily based on preventing its horizontal transmission and on the establishment of a vaccination programme with a live attenuated S48 strain (Toxovax™, MSD). The benefits are associated with protection against abortions induced by *T. gondii* during pregnancy and a decrease in tissue cyst development (Innes *et al.*, 2009; Verma and Khanna, 2013). Although the protective immunity against *T. gondii* can persist over one year, vaccination does not imply latent infections, and its use has two main drawbacks: a short shelf-life and a risk of infection to the humans who are handling live vaccines (Innes *et al.*, 2009; Verma and Khanna, 2013). In terms of efficacy, the design of successful vaccines requires blocking the stages of the life cycle (e.g., cell invasion, intracellular replication) and simulating the natural immune responses of the hosts (Zhang *et al.*, 2013). Therefore, vaccine development against *T. gondii* infection in humans and food-producing animals represents an adequate and global challenge that could greatly improve disease control. Regarding bovine neosporosis and vaccination, economic analyses suggest

that vaccination may be a more cost-effective and largely preferred approach than a test and cull programme (Reichel and Ellis, 2006). In the United States, a commercial killed vaccine for pregnant cows was available until 2009 (C548 NeoGuard™, Intervet), but it showed variable efficacy. At this time, no commercial vaccine is available, which precisely represents a major handicap that needs to be solved (Horcajo *et al.*, 2016). Live-attenuated vaccines appear to be the most promising approach, although subunit vaccines have also been investigated in relation to two functional points of view: the physical interaction between the parasite and its host cell during invasion or tachyzoite-to-bradyzoite stage conversion (Hemphill *et al.*, 2016), and these vaccines have emerged as the best way forward (Horcajo *et al.*, 2016).

Alternatively, several drugs appear to have a potentially beneficial effect on controlling these infections. Effective chemotherapy has been identified as an economically promising option in the literature (Häsler *et al.*, 2006a; Häsler *et al.*, 2006b). However, the disadvantage of using pharmacological treatments against toxoplasmosis and neosporosis is related to the timing of their administration; there is no evidence of infection until abortion cases are observed, and this timing could be indicative of a stage that is too late for treatment administration (Benavides *et al.*, 2014). An oral administration of drugs is expected to be effective against the *T. gondii* and *N. caninum* sporozoites released from oocysts. In addition, the drugs should be effective against the tachyzoite stage, which is associated with acute disease, and the bradyzoite stage, which occurs in the chronic phase of infection (Debache *et al.*, 2011). The ideal treatment would be parasiticidal against sporozoites and bradyzoites, but a parasitostatic capability against the tachyzoite stage, since tachyzoites have greater difficulty resisting the host cell's adaptive immune response. Additionally, a suitable drug should have low toxicity and should be able to cross the blood–brain barrier to effectively eliminate parasitic brain cysts (Neville *et al.*, 2015).

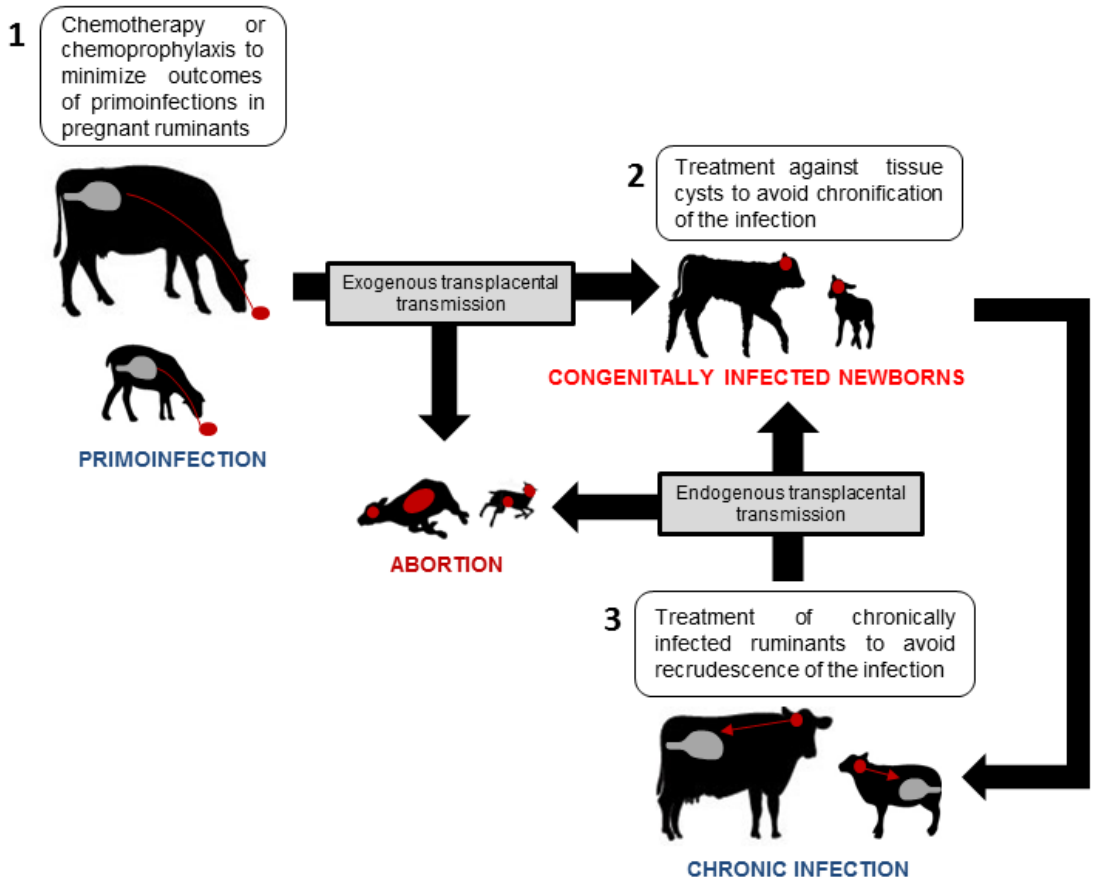
In practical terms, the treatment of ruminant toxoplasmosis and neosporosis should be focused on 3 main levels: i) chemotherapy or chemoprophylaxis in infected cows, ewes and goats (Figure 1), ii) treatment of newborn congenitally infected calves, lambs and kids (Figure 1) and iii) treatment of definitive hosts in order to reduce environmental contamination with *T. gondii* and *N. caninum* oocysts.

The comprehensive immune-chemotherapeutical approach with a combination of protective immunity generated by vaccines (applied before mating) and the capability of bioactive compounds (applied in the gestation period where abortions are more likely to occur) to limit the proliferation and dissemination of tachyzoites or to abrogate tissue cysts would be a great option for controlling toxoplasmosis and neosporosis in farm ruminants (Hemphill *et al.*, 2016). In a context where vaccination against both parasites needs to be improved, the objective of this review is to summarize relevant studies on the treatment of toxoplasmosis and neosporosis in farm ruminants that could serve as chemotherapeutical guidelines. Furthermore, in order to address novel therapeutic approaches, considerations on promising drugs will be described.

Available drug treatments for ruminant toxoplasmosis and neosporosis

Published research, including ruminant studies on drug therapy against toxoplasmosis (Table 1)

Figure 1- Intervention points for chemotherapy and chemoprophylaxis in toxoplasmosis and neosporosis in farm ruminants.



and neosporosis (Table 2), are reviewed. Each pharmacological group, with its chemical structure, main uses for therapy, mode/s of action, and *in vitro* and *in vivo* activities, are summarized.

Macrolide antibiotics

Macrolide antibiotics, which were discovered in the early 1950s, are a well-established class of antimicrobial agents containing 12- to 16-membered lactone rings that have been substituted with one or more sugar residues, some of which may be amino sugars (Schönfeld and Kirst, 2002). All macrolides inhibit bacterial protein synthesis to varying extents. The macrolides bind to the 50S ribosomal subunit with a specific target in the 23S ribosomal RNA molecule and various ribosomal proteins. The most recent hypothesis suggests that all macrolides stimulate the dissociation of peptidyl-tRNA from the ribosomes during the elongation phase, leading to the inhibition of protein synthesis (Brisson-Noël *et al.*, 1988; Mazzei *et al.*, 1993). The spectrum of activity includes many common pathogens, such as gram-positive bacteria (e.g., *Streptococcus pyogenes*, *Corynebacterium diphtheria* and *Staphylococcus aureus*), gram-negative bacteria (e.g., *Neisseria gonorrhoeae*, *Moraxella catarrhalis* and *Bordetella pertussis*) and protozoan parasites, including *T. gondii*, *Plasmodium falciparum* and *Entamoeba histolytica* (Root, 1999).

Spiramycin (2-[(4R,5S,6S,7R,9R,10R,11E,13E,16R)-6-[5-(4,5-dihydroxy-4,6-dimethyloxan-2-yl)oxy-4-(dimethylamino)-3-hydroxy-6-methyloxan-2-yl]oxy-10-[5-(dimethylamino)-6-methyloxan-2-yl]oxy-4-hydroxy-5-methoxy-9,16-dimethyl-2-oxo-1-oxacyclohexadeca-11,13-dien-7-yl]acetaldehyde), a 16-membered macrolide derived from *Streptomyces ambofaciens*, has been used for the treatment of

acute toxoplasmosis in pregnant women for decades because it is a safe and well-tolerated drug with no known adverse effects on the foetus (Desmonts and Couvreur, 1974; Daffos *et al.*, 1988; McCabe, 2001); however, its effectiveness and benefits are still unclear (Wallon *et al.*, 1999; Serranti *et al.*, 2011; Rodrigues *et al.*, 2014). Spiramycin showed limited *in vitro* activity against *T. gondii*, with a 50% inhibitory concentration (IC_{50}) of 20.16 µg/ml and an inhibitory, rather than a curative, effect (Chang and Pechere, 1988; Chamberland *et al.*, 1991). Despite its low *in vitro* efficacy, spiramycin was the first macrolide to demonstrate activity against acute toxoplasmosis in mice (Garin and Eyles, 1958). Spiramycin improves mouse survival after an acute *T. gondii* infection and reduces brain cyst burdens in both acute and chronic toxoplasmosis (Grujić *et al.*, 2005; Chew *et al.*, 2012). These paradoxical results are explained by spiramycin's ability to achieve intra-cellular and tissue concentrations that exceed its serum concentrations. Furthermore, spiramycin clearance is low, resulting in sustained tissue and intracellular concentrations (Smith, 1988).

Spiramycin has also been tested in pregnant ewes infected with *T. gondii* (RH strain) between the 85th and 100th days of pregnancy and treated orally (PO) with 100 mg/kg spiramycin from 3 weeks after infection until parturition. All ewes gave birth, with only one stillbirth in the untreated group. Analysis of the placental tissues did not show differences neither in the histopathological lesions, nor in the presence of *T. gondii* between the treated and untreated groups. However, the humoral immune response in pregnant ewes decreased in the treated group compared to that in the untreated group. Additionally, a lower number of lambs were seropositive to *T. gondii* in the treated group than in the untreated one. It was concluded that spiramycin treatment in ewes during the mid-stage of gestation exhibited a reduction in the humoral immune response in dams and in *T. gondii* seropositive lambs (Dubreuil, 1972).

Polyether ionophore antibiotics

Polyether ionophore antibiotics, whose structures involve an alkyl-rich, lipid-soluble exterior and a cage-like interior, are produced by *Streptomyces* spp. and form complexes with metal cations, transporting these complexes across hydrophobic membranes (Wang *et al.*, 2011). Polyether antibiotics form mainly neutral complexes with monovalent cations (i.e., monensin, salinomycin) or divalent metal cations (i.e., lasalocid, calcimycin) and with organic bases (i.e., lasalocid) (Westley, 1982; Bakker *et al.*, 1997; Rutkowski and Brzezinski, 2013). Polyether antibiotics induce ionic gradient perturbations, acting on different metabolic pathways (Couzinet *et al.*, 2000), and show broad spectrum bioactivity, including antibacterial, antifungal, antiparasitic and antiviral effects, as well as tumour cell cytotoxicity (Rutkowski and Brzezinski, 2013).Monensin (4-[2-[5-ethyl-5-[5-[6-hydroxy-6-(hydroxymethyl)-3,5-dimethyl-oxan-2-yl]-3-methyl oxolan-2-yl]oxolan-2-yl]-9-hydroxy-2,8-dimethyl-1,6-dioxaspiro[4.5]dec-7-yl]-3-methoxy-2-methyl-pentanoic acid) was shown to have coccidiostatic effects (Bergstrom and Maki, 1976; McDougald and Dunn, 1978) and growth promoting properties (Herberg *et al.*, 1978) by favouring ruminal propionic acid production (Richardson *et al.*, 1976). However, European Union regulations on the additives for use in animal nutrition banned the use of growth promoting agents as feed additives in animals (European Commission, 2005).

In vitro studies showed that polyether ionophore antibiotics affect all endogenous apicomplexan parasite forms, whether they are dividing or not (Couzinet *et al.*, 2000). Lasalocid (6-[(3R,4S,5S,7R)-7-[(2S,3S,5S)-5-ethyl-5-[(2R,5R,6S)-5-ethyl-5-hydroxy-6-methyloxan-2-yl]-3-methyloxolan-2-yl]-4-hydroxy-3,5-dimethyl-6-oxononyl]-2-hydroxy-3-methylbenzoic acid) at 0.05 µg/ml is directly toxic to extracellular *T. gondii* tachyzoites and inhibits cell penetration and

intracellular multiplication (Melton and Sheffield, 1975). Monensin at 0.001 µg/ml has also shown *in vitro* efficacy (IC_{50}) against *T. gondii* tachyzoites (Ricketts and Pfefferkorn, 1993; Rutkowski and Brzezinski, 2013). Working against the *T. gondii* cyst form, monensin inhibits the infectivity and viability of bradyzoites at low concentrations (0.1 ng/ml) (Couzinet *et al.*, 2000). Recently, it has been demonstrated that monensin, through TgMSH-1 protein, disrupts the mitochondrial function of *T. gondii*, triggering a disruption of cell cycle progression that precedes the events that resemble an autophagy-like death process (Lavine and Arrizabalaga, 2011; Lavine and Arrizabalaga, 2012). In addition, the disruption of mitochondrial function results in the generation of reactive oxygen species (Charvat and Arrizabalaga, 2016). *T. gondii* monensin resistance has been described after chemical mutagenesis *in vitro* (Ricketts and Pfefferkorn, 1993) or as a result of a disruption of TgMSH-1 (Garrison and Arrizabalaga, 2009). Furthermore, lasalocid and monensin at 0.001 ng/ml showed a 95% reduction of *N. caninum* tachyzoites in cell cultures (Lindsay *et al.*, 1994).

The efficacy of monensin against ovine toxoplasmosis was evaluated in experimentally infected pregnant sheep. Ninety-days pregnant ewes were experimentally infected PO with 2,000 and 12,000 *T. gondii* (M1 strain) oocysts and were treated PO with monensin (15 or 30 mg) daily from day 80 of gestation until parturition (Buxton *et al.*, 1988). Ewes receiving monensin showed a reduction in foetal mortality compared with the non-treated ewes (83.3% *vs* 44.8% alive lambs, respectively). In this study, monensin seems to act earlier in the infection, possibly by effects on the sporozoites released from infectious oocysts within the intestinal lumen. Ewes infected and treated with monensin showed a lesser febrile response. In addition, ewes receiving monensin showed lower anti-*T. gondii* IgG levels. However, when monensin administration ceased after lambing, circulating specific IgG antibodies against *T. gondii* increased over three months to reach values similar to those observed in infected and non-treated ewes. This observation suggests that monensin could also have a systemic effect, possibly acting on the tachyzoites present in the pregnant uterus. In addition, lambs born from ewes receiving monensin had higher live weights, and less of these animals showed evidence of infection and pathological changes in foetal or placental tissues than the lambs born from infected and non-treated ewes.

The possible systemic effect of monensin after *T. gondii* infection could be of value, as the time of infection during natural outbreaks of toxoplasmosis can only rarely be clearly defined. To avoid intestinal infection, and hence any intestinal effect of monensin PO at day 90 of pregnancy, the pregnant ewes were challenged subcutaneously (SC) with 100 *T. gondii* (M1 strain) tissue cysts (Buxton *et al.*, 1987). No differences in febrile and serological responses were found between the treated and non-treated groups. However, the treated ewes produced more viable lambs (less premature and greater live weight) than the untreated ewes (75% *vs* 42%, respectively). In addition, 58% of the lambs from treated ewes survived 72 hours after birth, whereas only 33% survived from the untreated ewes. Based on this evidence, it would appear that monensin, while being most effective in the gut lumen, does have a lesser systemic action, presumably by suppressing the multiplication of *T. gondii* in the placentome.

The accidental poisoning of domestic animals with monensin PO has been reported in cattle, horses, poultry and dogs (Beck and Harries, 1979; Wilson, 1980). The clinical findings in sheep include lethargy, stiffness,

Table 1 – Summary of published chemoprophylactic and chemotherapeutic studies against *T. gondii i*nfection in farm ruminants

Drug		Experimental design			Main results	References
Class	**Compounds**	**Animal**	**Infection**	**Treatment**		
Macrolide antibiotics	Spiramycin	85-100-days pregnant ewes (n=19)	1500 RH strain tachyzoites/kg body weight	100 mg/kg PO from 3 weeks after infection until parturition	Reduction in the humoral immune response in pregnant ewes and *T. gondii* seropositive lambs	Dubreuil, 1972
Polyether ionophore antibiotics	Monensin	80-days pregnant ewes (n=14)	100 M1 strain tissue cysts SC at day 90 of pregnancy	15 mg/day PO from day 80 of pregnancy until parturition	44% increase in live lambs and 43% enhancement in survival 72 hours after birth	Buxton *et al*., 1987
		80-days pregnant ewes (n=69)	2000-12000 M1 strain oocysts PO at day 90 of pregnancy	15-30 mg/day PO from day 80 of pregnancy until parturition	Reduction in febrile response, foetal mortality (46% increase in live lambs) and humoral immune response in dams and foetuses	Buxton *et al*., 1988
	Lasalocid	55-days pregnant ewes (n=33)	100 TS-1 strain oocysts PO at day 60 of pregnancy	30 g/day PO from day 55 of pregnancy until parturition	No effect in reducing humoral immune responses, lesions or abortions	Kirkbride, *et al*., 1992
Folate inhibitors	Sulphamezathine and pyrimethamine	89-days pregnant ewes (n=65)	2000 M3 isolate oocysts PO at 89 days of pregnancy	Sulphamezathine (83-166 mg/ kg SC). Pyrimethamine (1-2 mg/kg IP) on days 100, 115 and 130 of gestation, each of three consecutive days	No foetal mortality from 130 days of gestation. Reduction in antibody titres of lambs with less severe placental lesions	Buxton *et al*., 1993a
	Sulphadimidine	110-130-days pregnant ewes (n=200)	Field conditions: high abortion rate flock	Sulphadimidine 20-33 mg/kg IM, 4 times every 48 hours	Reduced abortion rate: 60% (before treatment) and 25% or 7% (after treatment)	Giadinis *et al*., 2011
Quinolones	Decoquinate	90-days pregnant ewes (n=98)	200 M3 isolate oocysts orally PO at 90 days of pregnancy	1-2 mg/kg PO from day 80 of pregnancy until parturition	Reduced febrile and humoral immune responses with increase of viable lambs up to 61.8%, increased weight of the lambs up to 33.3% and mean gestation period increased by 5 days	Buxton *et al*., 1996
Triazinones	Toltrazuril	Lambs (n=33)	$9x10^4$ IP and $1x10^4$ IM ME-49 oocysts	20-40 mg/kg PO twice, once every week, beginning 15 days pi	Reduction in serum antibody titres at 90 days after infection and 44.4% reduction in tissue cysts	Kul *et al*., 2013

PO: per os. Oral administration
SC: subcutaneously
IP: intraperitoneally
IM: intramuscularly
pi: post-infection

Table 2 – Summary of published chemoprophylactic and chemotherapeutic studies against *N. caninum* infection in farm

Drug		Experimental design			Main results	References
Class	**Compounds**	**Animal**	**Infection**	**Treatment**		
Polyether ionophore antibiotics	Monensin	Non-pregnant dairy cows (n=27)	$5x10^6$ Nc-1 tachyzoites SC	335 mg/day, bolus PO. From 21 days before challenge until 3 months pi	Lower humoral immune response on week 4 pi	Vanleeuwen *et al.*, 2011
Quinolones	Decoquinate	1.5-months pregnant heifers (n=77)	Chronically infected or primo-infected heifers	2 mg/kg PO from 1.5 until 8 months of pregnancy	Tendency to reduce abortions (44% in chronically infected heifers and 64% in primo-infected heifers) and infection in calves at birth (25% in chronically infected heifers and 40% in primo-infected heifers)	Journel *et al.*, 2012
Triazinones	Ponazuril	Calves (n=19)	$1x10^8$ IV and $1x10^8$ SC Nc-1 tachyzoites	20 mg/kg PO, applied 24 hours pi once or six consecutive days	Reduction of symptoms, lower antibody response and abrogation of parasite detection in organs	Kritzner *et al.*, 2002
	Toltrazuril	Calves (n=72)	Congenitally infected calves	20 mg/kg PO, three times, every second day, within 7 days after birth	Strong antibody reactivity four to six months after birth	Haerdi *et al.*, 2006
		Lambs (n=38)	Congenitally infected lambs	20 mg/kg PO on days 0, 7, 14 and 21 after birth	Lower antibody levels, but no reduction of presence or severity of histopathological lesions	Syed-Hussain *et al.*, 2015a
Triazinones + Folate inhibitors	Toltrazuril + Sulphadiazine and Trimethoprim	Dairy cows (n=936)	Field conditions: high abortion rate herds	Toltrazuril 20 mg/kg/day IV for 3 consecutive days to newborn calves during the first week. Sulphadiazine/trimethoprim at 20 mg/kg IV once a year from 3 months of age	Reduction of abortions (from 188 to 9)	Cuteri *et al.*, 2005

PO: per os. Oral administration
SC: subcutaneously
IV: intravenously
pi: post-infection

muscular weakness, a stilted gait and recumbency, followed by a decrease in the muscle volume of the rump and thigh. The post-mortem lesions in the skeletal muscles consisted of pale streaking, with atrophy in the chronic stages. In lambs younger than one month old, diffuse gastrointestinal haemorrhage was the only finding (Nation *et al.*, 1982). Later, another researcher reported an outbreak of monensin poisoning in sheep and highlighted the need for a licensed, safe product (Synge, 1989). An option to avoid monensin poisoning in sheep would be to use blocks containing monensin or slow-release boluses. In addition, this option would overcome the practical problems of monensin delivery to grazing sheep (Trees, 1989; Ellis and Costigan, 1990).

Lasalocid has also been tested for its efficacy against ovine toxoplasmosis (Kirkbride *et al.*, 1992). Ewes were treated PO with lasalocid (30 g/day) daily from day 55 of pregnancy until lambing and were PO inoculated with 100 *T. gondii* (TS-1 strain) oocysts 5 days after beginning lasalocid administration. Similar specific antibody titres and histopathological lesions were found in the ewes and foetuses, and there were no differences in the rate of abortion and neonatal mortality in both the treated and untreated ewes. These results suggest that lasalocid was not effective in preventing *T. gondii* abortion in sheep.

Concerning *N. caninum* infection in cattle, a risk factor analysis in dairy farms showed that cows receiving monensin as a feed additive were 1.5 times less likely to be infected with this parasite than the cows that did not receive monensin (Vanleeuwen *et al.*, 2010). Thus, the effect of monensin was tested against experimental neosporosis in cattle (Vanleeuwen *et al.*, 2011). Non-pregnant cows were treated with a slow-release bolus PO that delivered 100 days of monensin (335 mg/day) and were challenged with $5x10^6$ *N. caninum* (Nc-1 strain) tachyzoites by the SC route three weeks after bolus administration. The cows treated with monensin showed a significantly lower humoral immune response than those treated with a placebo at week 4 post-challenge. Before recommendations on monensin use for neosporosis could be made, further research on other larger populations of cattle, preferably pregnant, must be explored. Furthermore, trials on monensin's effectiveness in more natural modes of *N. caninum* transmission (e.g., bradyzoite recrudescence leading to transplacental transmission, or oocyst ingestion) would also be needed.

In summary, PO administered monensin was shown to be partially effective in the control of ovine toxoplasmosis, but it is not licensed for use in the EU and may be toxic at high dosages. In contrast, PO dosed lasalocid did not exhibit efficacy in reducing the ovine toxoplasmosis outcome. In *N. caninum*-infected non-pregnant cows, monensin was associated with a lower specific humoural immune response, but further research on the outcome of infection in pregnant animals is needed.

Folate inhibitors

Folate inhibitors, such as sulphonamides and pyrimethamine, and their mechanism of action were identified years ago (Hitchings and Burchall, 1965). Sulphonamides are para-aminobenzoic acid analogues that competitively inhibit parasite dihydropteroate synthetase (DPHS). Pyrimethamine is a folate analogue that competitively inhibits parasite dihydrofolate reductase (DHFR). DHPS is one of the enzymes responsible for folate compound synthesis while DHFR maintains folate in a reduced state. Folate compounds are essential for parasite metabolism, including nucleoside biosynthesis and therefore nucleic acid formation. Sulphonamides, in combination with pyrimethamine, are a mainstay of chemotherapy in human toxoplasmosis (Montoya and Liesenfeld, 2004).

Sulphonamides, such as sulphadiazine (IC_{50} of 2.5 µg/ml), were found to have important inhibitory effects on *T. gondii*. This inhibitory effect on parasite growth was associated with a reduction in the number of parasitized cells and intracellular parasites that were morphologically normal (Derouin and Chastang, 1989).

In contrast, sulphonamides demonstrate little activity against *N. caninum* tachyzoites at 100 µg/ml (Lindsay *et al.*, 1994). With DHFR inhibitors, a strong inhibition of *T. gondii* growth was observed with pyrimethamine (IC_{50} of 0.04 µg/ml) and trimethoprim (IC_{50} of 2.3 µg/ml), along with striking morphological changes in the parasites. Additionally, trimethoprim is effective against *N. caninum* tachyzoites at 10 µg/ml (Lindsay *et al.*, 1994). When sulphonamides and DHFR inhibitors were used in combination, a remarkable synergistic activity against the replicating forms of *T. gondii* and *N. caninum* was observed (Derouin and Chastang, 1989; Lindsay *et al.*, 1996). Laboratory-induced resistance to antifolates has been described for *T. gondii* and *N. caninum* (Pfefferkorn *et al.*, 1992; Lindsay *et al.*, 1996). In addition, it is also known that sulphonamide resistance in *T. gondii* can be associated with nucleotide polymorphism in DHPS (Aspinall *et al.*, 2002). When pyrimethamine and sulphadiazine were administered in combination to non-pregnant mice for 10 days from day 1 after intraperitoneal (IP) infection with *T. gondii* tachyzoites, 100% of the mice survived, and they were considered cured because the parasites remained undetectable (Piketty *et al.*, 1990). Likewise, sulphadiazine is effective in preventing deaths and clinical disease in *N. caninum* infected mice (Lindsay and Dubey, 1990a).

Experimentally induced toxoplasmosis in pregnant ewes with 2,000 oocysts (M3 strain) at 89 days of pregnancy was treated with a combination of sulphamezathine and pyrimethamine sulphate for three consecutive days on days 100, 115 and 130 of gestation. Sulphamezathine (1 g per 3 ml of solution) was injected SC at an initial dose of 5 ml/10 kg on the first day, with subsequent doses on the following two days of 2.5 ml/10 kg. Pyrimethamine sulphate (10 mg/ml) was injected IP at 2 mg/kg on the first treatment day and at 1 mg/kg on the two subsequent days. After 130 days of gestation, 30% of foetuses from untreated ewes died, whereas all the lambs from the treated ewes were born and were viable. In addition, the gestational period of infected and untreated ewes was shorter than that observed in the infected and treated ewes. At birth, all the lambs showed a specific antibody response to the protozoa, indicating that the infection was in utero. However, the mean IFAT titres of lambs born from the infected and treated ewes were lower than those of the lambs born from the infected and untreated ewes (1/256 *vs* 1/730 for IgG and 1/665 vs 1/5206 for IgM). Histopathological examination showed less severe placental lesions in the lambs born from the treated ewes (Buxton *et al.*, 1993a).

In field conditions, the therapeutic efficacy of sulphonamides in an ovine toxoplasmosis outbreak has been evaluated. Before treatment, 60% of the ewes in the flock had aborted, and treatment with sulphadimine at 20 mg/kg intramuscularly (IM), 4 times every 48 hours, reduced the abortion rate to 25%; the rest of the ewes gave birth normally, but 61.1% of their lambs were stillbirths and did not survive. A higher dosage at 33 mg/kg IM, 4 times every 48 hours, reduced the abortion rate to 7%, and 75% of the lambs survived (Giadinis *et al.*, 2011). Folate inhibitors have also been evaluated in field conditions for the treatment of neosporosis in cattle. A combination of toltrazuril given intravenously (IV) at 20 mg/kg/day for 3 consecutive days to newborn calves during the first week of age and sulphadiazine/trimethoprim given IV at 20 mg/kg to cattle once a year from 3 months of age along with dog treatment with toltrazuril and periodic disinfection of environment

resulted in a significant reduction of abortion (from 188 to 9) (Cuteri *et al.*, 2005).

In brief, parenteral administration of sulphonamides, in combination with pyrimethamine, could be a valuable option for chemotherapy of ovine toxoplasmosis in the third term of gestation, as a reduction in abortion rates, but not in the percentage of transplacental transmission, is observed. There is some evidence in field experiments that folate inhibitor administration can reduce abortions in ruminants, although more research is needed.

Quinolones

Quinolones were derived from quinine [112]. Decoquinate (6-ethyl-(decycloxy)-7-ethoxy-4-hydroxy-3-quinolinecarboxylate) is a quinolone derivative coccidiostat that was initially developed as an anticoccidial for poultry in 1967 (Williams, 2006). Decoquinate's effects are mainly observed earlier in the life cycle of coccidia by acting on the sporozoites that are released from the ingested sporulated oocysts during the first day of their life cycle (Joyner and Norton, 1971; Fitzgerald and Mansfield, 1986). Decoquinate significantly inhibits mitochondrial respiration and electron transport in *Eimeria* (Fry and Williams, 1984).

It has been used for over 20 years in the control of coccidiosis in domestic ruminants (Laval and Remy, 1994; Daugschies and Najdrowski, 2005). Decoquinate is registered and commercialized for use in ruminants in multiple countries worldwide, including the USA and several countries in Latin America, Europe and the Middle East (Taylor and Bartram, 2012). Decoquinate is poorly absorbed by the target species when administered PO and is largely eliminated unchanged in faeces. A recent study in cattle showed that only small quantities of decoquinate become systemically available and that the drug is rapidly eliminated with negligible residues in milk (Quintero-de Leonardo *et al.*, 2009). Therefore, decoquinate is in Annex II of the EU Council Regulation No.2377 / 90 (European Commission, 1994), which means that it is not subject to maximum residue levels, with a meat withdrawal time of zero days in cattle and sheep in Europe (EMEA, 2000).

Research has shown decoquinate to have *in vitro* activity against *T. gondii* tachyzoites with an IC_{50} of 0.005 µg/ml (Ricketts and Pfefferkorn, 1993), although chemical mutagenesis revealed *T. gondii* resistant mutants (Pfefferkorn *et al.*, 1993; Ricketts and Pfefferkorn, 1993). Likewise, intracellular *N. caninum* tachyzoites were killed quickly at 0.1 µg/ml (Lindsay *et al.*, 1997).

In a study, sheep were challenged PO with 200 *T. gondii* (M3 strain) oocysts at 90 days of gestation, and decoquinate was administered PO at 1 or 2 mg⁄kg⁄day from 10 days prior to oocyst challenge until lambing (Buxton *et al.*, 1996). The administration of decoquinate at the higher rate of 2 mg⁄kg⁄day was associated with a delayed onset of the febrile response to infection, reduction in the overall severity of fever and a delay in the production of specific antibodies to the parasite. This treatment also reduced the placental damage caused by the protozoa, lengthened the mean gestation period by five days and increased the proportion of viable lambs (up 61.8%) and the mean weight of the lambs (up 33.3%) in comparison with ewes that were not treated with decoquinate but were challenged with *T. gondii* oocysts.

In addition, it is necessary to note that the decoquinate-medicated feed distributed to pregnant heifers that are chronically infected or primo-infected with *N. caninum* at the dose of 2 mg/kg from 1.5 months of gestation until the end of the 8th month of pregnancy tends to reduce the associated abortions (from 38% to 21% in chronically infected heifers and from 17% to 6% in primo-infected heifers) and neonatal infections, as more seronegative calves were born (28% in the treated group *vs* 21% in the untreated group for chronically

infected heifers and 59% in the treated group *vs* 35% in the untreated group for primo-infected heifers) (Journel *et al*., 2002)

To summarize, PO administration of decoquinate reduces the effect of experimentally induced toxoplasmosis in pregnant ewes. These results support the indication of decoquinate as an aid in the prevention of abortion due to ovine toxoplasmosis if used during mid and late pregnancy. Decoquinate may have an application in the treatment of bovine neosporosis.

Triazinones

The triazine structure is a heterocyclic ring, analogous to the six-membered benzene ring, but with three carbons replaced by nitrogen. The three isomers of triazine are distinguished from each other by the positions of their nitrogen atoms and are referred to 1,2,3-triazine, 1,2,4-triazine and 1,3,5-triazine (Arshad *et al.*, 2015).

Toltrazuril, a symmetric 1,3,5-triazine derivative, is metabolized into an active metabolite, toltrazuril sulphone (ponazuril). Both toltrazuril and ponazuril are effective against a broad spectrum of cyst-forming and non-cyst-forming apicomplexan protozoa (Haberkorn, 1996). Several studies have shown that toltrazuril and ponazuril affect the respiratory chain through the reduction of some enzymes of the respiratory chain, such as succinate-cytochrome C reductase, NADH oxidase and succinate oxidase. Second, the DHFR involved in pyrimidine synthesis is also affected, but the effect is weaker than that observed with pyrimethamine (Harder and Haberkorn, 1989). In addition, nuclear division is affected, mitochondrial activity is impaired, and the endoplasmic reticulum is vacuolized (Mehlhorn *et al.*, 1984). Additionally, differential phenotypic changes between the tachyzoites of *N. caninum* and *T. gondii* were observed after ponazuril treatment (Mitchell *et al.*, 2005).

In vitro, toltrazuril has positive effects against *T. gondii* with an IC_{50} of 0.4 µg/ml (Ricketts and Pfefferkorn, 1993; Greif *et al.*, 2001; Mitchell *et al.*, 2004). Toltrazuril also shows *in vitro* efficacy against *N. caninum*, although a concentration of 30 µg/ml for 14 days is required to eliminate parasites (Darius *et al.*, 2004), and no NcGRA2 expression is found, showing a lack of parasite viability after treatment (Strohbusch *et al.*, 2008). Ponazuril administered prophylactically or after treatment in mice is effective in preventing acute toxoplasmosis based on a lack of mortality (Mitchell *et al.*, 2004). Toltrazuril and ponazuril administered in *N. caninum*-infected non-pregnant mice abrogate the formation of cerebral lesions, and 90% of the treated mice had *N. caninum*-DNA-free brains (Gottstein *et al.*, 2001). In addition, toltrazuril reduces foetal losses and transplacental transmission in *N. caninum*-infected pregnant mice (Gottstein *et al.*, 2005), and it has an impact on the course of infection in congenitally *N. caninum*-infected newborn mice (Strohbusch *et al.*, 2009a).

It has been emphasized that the production of *T. gondii* free lamb, sheep or goat meat for human consumption is critically important for public health (Kijlstra and Jongert, 2009). The *in vivo* therapeutic efficacy of toltrazuril on *T. gondii* tissue cysts in experimentally infected lambs has been studied after a chronic infection in newborn lambs through a parenteral inoculation of 10^5 *T. gondii* (ME-49 strain) oocysts (Kul *et al.*, 2013). Beginning at the 15th day after inoculation, the lambs were treated with toltrazuril PO 2 times, once every week at a dose of 20 mg/kg and 40 mg/kg. Following toltrazuril treatment, at day 90 after inoculation, the specific immune humoral response in lambs of both treatment groups was lower. On day 90 after inoculation, the lambs were necropsied. The histopathological findings in the toltrazuril-treated lambs include morphologic and structural changes of the tissue cysts in the musculature, which were characterized by initial degenerative changes in

the cyst wall and a minimal inflammatory cell response. The presence of *T. gondii* DNA in heart, brain and semitendinosus muscle from the treated groups was lower than that in tissues of the non-treated lambs. Moreover, in the treated groups, 4 out of 9 (44.4%) lambs did not contain any tissue cysts in the examined tissues, but untreated animals showed *T. gondii* tissue cysts at least in one of the sampled muscles. The administration of toltrazuril seems to be associated with a reduction of *T. gondii* cysts in the musculature.

It is doubtful that encysted *N. caninum* bradyzoites were susceptible to toltrazuril treatment. Congenitally infected calves born from *Neospora*-seropositive cows were PO treated with toltrazuril at 20 mg/kg three times at 48-hour intervals within 7 days after birth, following a humoral immune response (Haerdi *et al.*, 2006). Four to six months after birth, a stronger antibody reactivity was found in the treated animals than in the untreated calves. Conversely, in a study to evaluate whether the treatment of congenitally infected lambs with toltrazuril PO at 20 mg/kg on days 0, 7, 14 and 21 after birth eliminated *N. caninum*, toltrazuril did not show any effect on the reduction of *N. caninum* presence or on the severity of histopathological lesions, and the lambs were all seropositive, although they had significantly lower specific antibody levels than those in the untreated animals, suggesting higher antigenic stimulation in the non-treated lambs than in the treated lambs (Syed-Hussain *et al.*, 2015a).

The efficacy of ponazuril has been tested in calves that were experimentally infected with *N. caninum* at $2x10^8$ tachyzoites (Nc-1 strain) (Kritzner *et al.*, 2002). Medication was performed PO with 20 mg/kg of ponazuril. The first medication dose was applied 24 hours after infection, and if repeated, it was administered every subsequent 24 hours for six days. Ponazuril allows a complete abrogation of parasite DNA detection by PCR in the brain and other organs. Regarding the non-medicated calves, it is noteworthy that there was a relatively lower susceptibility of calves to experimental infection, since only 50% of the calves became PCR-positive in the brain and muscles. The efficacy of a six-day treatment was also suggested by the significantly lower anti-*N. caninum* antibody response and later seroconversion than those in the infected and non-medicated calves.

In addition, as explained above, toltrazuril administration to newborn calves, along with sulphadiazine/trimethoprim administration, resulted in reduced abortions and *N. caninum* seroprevalence in naturally infected cattle herds (Cuteri *et al.*, 2005).

In summary, toltrazuril PO administered in lambs would be a valuable strategy to minimize human exposure to *T. gondii* tissue cysts from the consumption of raw or undercooked mutton. Triazinon derivatives are directed against an *N. caninum* tachyzoite challenge, whereas treatment of congenitally infected young ruminants remains an elusive goal. In addition, it would likely result in considerable unacceptable milk or meat residues or withdrawal periods (Dubey *et al.*, 2007).

Present approaches in drug development for ruminant toxoplasmosis and neosporosis

Experimental studies have revealed that several compounds have potentially interesting effects on *T. gondii* and *N. caninum in vitro*, but only a few drugs have been tested in laboratory animal models *in vivo*. The most interesting drugs tested for toxoplasmosis and neosporosis were derived from screenings in other intracellular protozoan parasites, including *Plasmodium*, *Trypanosoma* and *Leishmania* species, and some drugs exhibited broad-spectrum anti-parasitic activity against various protozoan and helminth parasites. However, it is notable that *T. gondii* was the least responsive to these sets of drugs, suggesting that it may be more difficult to target chemotherapeutically than the aforementioned parasites (Guiguemde *et al.*, 2010). In addition,

other approaches identified compounds that inhibited targets that were conserved almost exclusively within the group of apicomplexan parasites; thus, drug repurposing is a valuable option (Brown and Superti-Furga, 2003; Hemphill *et al.*, 2016). The main prospective drug classes with *in vitro* and *in vivo* activity in small animal models against *T. gondii* and *N. caninum* are reviewed below (Table 3), although more chemotherapeutic options for toxoplasmosis and neosporosis have been described (Neville *et al.*, 2015; Hemphill *et al.*, 2016).

Thiazolides

Nitazoxanide (2-acetolyloxy-N-(5-nitro 2-thiazolyl) benzamide), the mother compound of this class, is essentially composed of a nitrothiazole-ring and a salicylic acid moiety, which are linked together through an amide bond (Hemphill *et al.*, 2006; Hemphill *et al.*, 2007). Since nitazoxanide non-selectivity can lead to undesired side effects in both human and animals, nitazoxanide derivatives were designed without the undesirable nitro group (Esposito *et al.*, 2007b). The nitazoxanide derivative RM4847 produces the upregulation of NQO1 (quinone reductase) expression by *N. caninum* but not by *T. gondii*. This fact may reflect differences between these parasites in terms of the mechanisms of circumventing host cell apoptosis (Muller and Hemphill, 2011). Thiazolides have favourable effects against *N. caninum in vitro*, with an IC_{50} of 4.23 µM and 13.68 µM for nitazoxanide and RM4847, respectively. Host cell invasion of extracellular *N. caninum* tachyzoites is inhibited by RM4847, but not by nitazoxanide (Esposito *et al.*, 2007a), and RM4847 was shown to be much more effective against *T. gondii* tachyzoites, with an IC_{50} of 0.2 µM (Müller *et al.*, 2009). For the treatment of *N. caninum* in mice, nitazoxanide fails when it is applied PO, or it is even toxic when applied IP (Debache *et al.*, 2011). In calves, the use of nitazoxanide to treat *Cryptosporidium* infection showed acute diarrhoea in non-infected animals, which indicates that this compound severely affects the intestinal flora (Schnyder *et al.*, 2009). In conclusion, thiazolides might be interesting tools to study the biology of *N. caninum* and possibly *T. gondii*, but they are useless as drugs since they exert acute toxicity in small and large animals.

Diamidines

Diamidines represent a class of broad-spectrum antimicrobial compounds in which pentamidine and its analogues exhibit activity against intracellular and extracellular protozoan parasites (Wilson *et al.*, 2008; Buckner and Navabi, 2010). Pentamidine displays *in vitro* activity against *T. gondii* by inhibiting the replication of the parasites in cell cultures (Lindsay *et al.*, 1991).

Pentamidine derivatives, and the more recently synthesised di-cationic arylimidamides, which have more favourable pharmacokinetic profiles, improved bioavailability, lowered host toxicity and had a higher chance of passing the blood-brain barrier to exert its activity by binding to AT-rich sites in the DNA minor groove, thus inhibiting transcription or the interaction with DNA-binding enzymes, such as topoisomerases or nucleases (Wilson *et al.*, 2008). This result indicates that these compounds could influence gene expression, and thus many diverse cellular functions could be affected. Di-cationic arylimidamides, such as DB786, DB750 and DB745, showed *in vitro* activity against *T. gondii* tachyzoites, with an IC_{50} of 0.22 µM, 0.16 µM and 0.03 µM, respectively (Leepin *et al.*, 2008; Kropf *et al.*, 2012). In contrast to DB750, DB745 also had a profound negative impact on extracellular *T. gondii* tachyzoites. In addition, a lower adaptation of *T. gondii* tachyzoites to DB745 was observed (Kropf *et al.*, 2012). The lowest IC_{50}s against *N. caninum* tachyzoites were found for DB786, DB750 and DB745 (0.21 µM, 0.23 µM, 0.08 µM, respectively), which caused damage to the parasite's ultrastructure.

The activities of DB750 and DB786 are limited to intracellular *N. caninum* tachyzoites; however, DB745 had an impact on both host cell invasion and intracellular proliferation (Leepin *et al.*, 2008; Schorer *et al.*, 2012). Di-cationic arylimidamides have also been found to be effective against neosporosis in mice. DB750 that was administered IP prior to infection or 14 days after infection reduced the severity of clinical signs and the cerebral parasite load, while PO application resulted in weight loss, indicating toxicity (Debache *et al.*, 2011; Schorer *et al.*, 2012). In addition, DB745 IP treatment initiated 14 days after infection had similar positive effects on the percentage of surviving mice and the parasite load in the brain (Schorer *et al.*, 2012). Arylimidamide treatments in mice beginning 14 days post-infection, after the *N. caninum* tachyzoites had crossed the blood-brain border and invaded the central nervous system (CNS) (Collantes-Fernández *et al.*, 2006a), indicated that DB745, just like DB750, most likely crossed the blood–brain barrier and also exerted its action within the cerebral tissues (Debache *et al.*, 2011; Schorer *et al.*, 2012). Potentially, features of DB745 could open the door for testing this compound against neosporosis in ruminants.

Artemisinins

Chinese herbal extracts are thought to possess the desired properties of potency and low toxicity, and they show promise for the identification of new therapeutic agents. Artemisinin, a sesquiterpene lactone with an unusual endoperoxide bond in a unique 1,2,4-trioxane heterocycle [3R,5aS,6R,8aS,9R,12S,12aR)-octahydro-3,6,9-trimethyl-3,12-epoxy-12H-pyrano[4,3-j]-1,2-benzodioxepin-10(3H)-one] is present in the leaves and flowers of the sweet wormwood (*Artemisia annua*) (Bilia *et al.*, 2006), and its derivatives are highly potent antimalarial drugs. This safe drug class is also effective against apicomplexan parasites causing abortions in ruminants such as *T. gondii* and *N. caninum*. The mechanism of action of artemisinin and its derivatives is dependent upon the presence of the endoperoxide bridge (Haynes and Krishna, 2004), although the difference in potencies between *P. falciparum* and *T. gondii* suggests that different targets may be affected in these two organisms (Dunay *et al.*, 2009). Additionally, it is known that artemisinin perturbs calcium homeostasis in *T. gondii*, supporting the idea that Ca_2-ATPases (SERCA) are potential drug targets in parasites (Nagamune *et al.*, 2007). Artemisinin, when administered *in vitro* at 0.4 µg/ml for 5 days or 1.3 µg/ml for 14 days, completely eliminated *T. gondii* (Ke *et al.*, 1990). Artesunate showed an IC_{50} of 0.075 µM (Gomes *et al.*, 2012). Artesunate and its active metabolite, dihydroartemisinin, resulted in approximately 40% and 70% growth inhibition *in vitro*, respectively, and the combination resulted in approximately 65% inhibition. As they are able to cross the blood-brain barrier, *in vivo* experiments with low virulence *T. gondii* (DUR strain) challenge causing a chronic infection in mice, different from previous artemisinin derivatives studies with RH strain, showed a 40% reduction in the number of *T. gondii* tissue cysts found in the brain of mice treated 5 days with artesunate-dihydroartemisinin and also modifications in the microscopic aspect of the cysts (Sarciron *et al.*, 2000). Artemisone, a second-generation semi-synthetic *artemisinin derivative, and artemiside, the* thiomorpholine precursor of *artemisone*, with an improved half-live, oral bioavailability, metabolic stability in various animal systems (Haynes *et al.*, 2006), tolerance *in vivo* (Nagelschmitz *et al.*, 2008) and a lack of detectable neurotoxic potential (Nontprasert *et al.*, 1998; Schmuck *et al.*, 2009), are the most potent artemisinin analogues to date in terms of inhibiting the growth of *T. gondii in vitro*, with an IC_{50} of 0.12 µM and 0.10 µM for artemisone and artemiside, respectively. Both of these compounds (artemiside and

Table 3 – Summary of published research, including *in vitro* and *in vivo* laboratory animal studies, on prospective drugs against toxoplasmosis and neosporosis

Drug		*In vitro* results	References	*In vivo* results from small animal studies	References
Class	**Compounds**				
Thiazolides	Nitazoxanide and nitazoxanide derivatives	Nitazoxanide and RM4847 showed activity against *N. caninum*. RM4847 is effective against *T. gondii*	Esposito *et al*., 2007a Müller *et al*., 2009	Nitazoxanide in mice PO is not effective in a mouse model of neosporosis and IP shows high toxicity	Debache *et al*., 2011
Diamidines	Di-cationic arylimidamides	DB786, DB750 and DB745 inhibit proliferation of *T. gondii* and *N. caninum*	Leepin *et al*., 2008 Kropf *et al*., 2012 Schorer *et al*., 2012	DB750 and DB745 administered IP reduce mortality and brain parasite load in mouse neosporosis.	Debache *et al*., 2011 Schorer *et al*., 2012
Artemisinins	Artemisinin derivatives	Amongst all the tested artemisinin derivatives, artemisone and artemiside are *the most potent against T. gondii. In addition, artemisone is also effective against N. caninum*	Ke *et al*., 1990 Sarciron *et al*., 2000 Kim *et al*., 2002 Dunay *et al*., 2009 Mazuz *et al*., 2012 Müller *et al*., 2015b Qian *et al*., 2015	Artesunate-dihydroartemisinin (PO) showed a reduction in the burden of brain cysts and artemisone and artemiside (SC) are effective against acute, chronic and reactivated toxoplasmosis. Controversial results are found on the efficacy of artemisone in gerbil (IP) and mouse (PO) models of neosporosis	Sarciron *et al*., 2000 Dunay *et al*., 2009 Mazuz *et al*., 2012 Müller *et al*., 2016
Naphthoquinones	Atovaquone	Active against *T. gondii* tachyzoites and, at high concentrations, also bradyzoites	Araujo *et al*., 1991 Huskinson-Mark *et al*., 1991 Araujo *et al*., 1992 Romand *et al*., 1993 Meneceur *et al*., 2008	Atovaquone PO is efficient for the treatment of acute and chronic toxoplasmosis. Additionally, atovaquone IV and PO treatment is effective against reactivated mouse toxoplasmosis	Araujo *et al*., 1991 Araujo *et al*., 1992 Romnd *et al*., 1993 Ferguson *et al*., 1994 Dunay *et al*., 2004
	Buparvaquone	Slow inhibition of *N. caninum* tachyzoites and adaptation to high levels of buparvaquone	Müller et al., 2015a	Buparvaquone PO or IP is effective in non-pregnant and pregnant mouse model of neosporosis	Müller *et al*., 2015a Müller *et al*., 2016
Anticancer drugs	Miltefosine	Efficacious against *T. gondii* extracellular tachyzoites and *N. caninum* tachyzoites proliferation	Debache *et al*., 2012 Eissa *et al*., 2015	Miltefosine PO reduces brain cyst burden and pathological lesions in chronic mouse toxoplasmosis. Moreover, miltefosine PO improves survival and reduces brain parasite burden in a mouse model of neosporosis	Debache *et al*., 2012 Eissa *et al*., 2015
	Organometallic ruthenium complexes	Efficacious against *T. gondii* and *N. caninum* in the nanomolar range	Barna *et al*., 2013 Basto *et al*., 2017	No studies available	

Table 3 – Continued

Drug		*In vitro* results	References	*In vivo* results from small animal studies	References
Class	**Compounds**				
Endochin-like quinolones	4-(1H)-quinolone derivatives	Nanomolar concentrations of ELQ-271 and ELQ-316 against *T. gondii* and ELQ-400 against *N. caninum* were effective	Dogget *et al*., 2012 Müller *et al*., 2017a	ELQ-271 and ELQ-316 were effective against acute toxoplasmosis in mice when administered PO and against the cyst form of *T. gondii* in mice when administered IP. ELQ-400 was effective PO in experimentally *N. caninum* infected non-pregnant mice	Dogget *et al*., 2012 Müller *et al*., 2017a
Calcium-dependent protein kinase inhibitors	Bumped kinase inhibitors (BKIs)	BKI-1517, BKI-1294 and BKI-1553 showed efficacy against *N. caninum* and BKI-1294 is effective against *T. gondii*.	Ojo *et al*., 2014 Winzer *et al*., 2015 Müller *et al*., 2017b	BKI-1294 and BKI-1517 PO led to good protection against vertical transmission in a pregnant mouse model of neosporosis. Additionally, BKI-1294 reduces *T. gondii* tachyzoites in an acute mouse model of toxoplasmosis and is effective in a murine vertical transmission model of *T. gondii*	Doggett *et al*., 2014 Ojo *et al*., 2014 Winzer *et al*., 2015 Müller *et al*., 2017b

PO: per os. Oral administration
SC: subcutaneously
IP: intraperitoneally
IV: intravenously

artemisone) were effective in reducing mortality during an acute challenge (60% of artemiside-treated mice and more than 50% of artemisone-treated mice survived) and during the reactivation of chronic infection in a mouse model (80% of artemiside-treated mice and 60% of artemisone-treated mice). Furthermore, there was an accompanying reduction in the chronic burden of tissue cysts in the CNS (Dunay *et al.*, 2009).

Against *N. caninum*, artemisinin reduces the intracellular multiplication of tachyzoites at ≥0.1 µg/ml for 30 hours or 1 µg/ml for 14 days (Kim *et al.*, 2002), and artemether exhibited activity against tachyzoite replication with an IC_{50} of 1.0 µg/ml (Qian *et al.*, 2015). Additionally, artemisone, when added prior to infection or in established infection, reduced the number of *N. caninum*-infected cells (Mazuz *et al.*, 2012) with an IC_{50} of 3 nM, exerting their activity against intracellular parasites, causing ultrastructural alterations and switched aberrant gene expression, including bradyzoite markers (Muller *et al.*, 2015b). In a gerbil model for acute neosporosis, artemisone increased survival, as 1 out of 8 mice died in the artemisone-treated group, while 8 out 9 mice succumbed in the control group (Mazuz *et al.*, 2012). However, in a mouse model of neosporosis, artemiside and artemisone had no effect on parasite loads in the brain or in the lungs (Müller *et al.*, 2016). In short, artemiside seems to be useful against *T. gondii in vitro* and in mice models whereas artemisone is less likely to be promising for neosporosis in ruminants.

Naphthoquinones

Naphthoquinone is a class of organic compounds derived from naphthalene. Buparvaquone (2-((4-tert-butylcyclohexyl)methyl)-3-hydroxy-1,4-naphthoquinone) and atovaquone (trans-2[4-(4-chlorophenyl) cyclohexyl]-3-hydroxy-1,4-naphthalenedione) are hydroxynaphthoquinones related to parvaquone and were originally developed as anti-malarial compounds (Hudson *et al.*, 1985; Hudson *et al.*, 1991).

Buparvaquone is currently commercially available for use in endemic regions against theileriosis in cattle (Wilkie *et al.*, 1998); however, in other regions of the world, e.g., the EU, it is not registered. A focus on the parasite mitochondrion as an anti-parasitic target has been a priority in the drug development field for decades (Mather and Vaidya, 2008; Sen and Majumder, 2008). Hydroxynaphthoquinones are structurally similar to the inner mitochondrial protein ubiquinone (also called coenzyme Q), which is an integral component of electron flow in aerobic respiration. Ubiquinone accepts electrons from the dehydrogenase enzymes and passes them to the electron transport cytochromes (Sun *et al.*, 1992). The passage of electrons from ubiquinone to the cytochrome *bc*1 (complex III) requires the binding of coenzyme Q complex III at the Qo cytochrome domain; it is this step that is inhibited by hydroxynaphthoquinones (Fry and Pudney, 1992; Pfefferkorn *et al.*, 1993; McFadden *et al.*, 2000). The consequence of this inhibition is the collapse of the mitochondrial membrane potential (Srivastava *et al.*, 1997). On the one hand, atovaquone inhibited the replication of *T. gondii* tachyzoites *in vitro* with an IC_{50} of 64 nM (Romand *et al.*, 1993; Meneceur *et al.*, 2008). Moreover, atovaquone is active *in vitro* against the cyst stage of *T. gondii* (Huskinson-Mark *et al.*, 1991), although high concentrations of it are required (Araujo *et al.*, 1991; Araujo *et al.*, 1992). In a mouse model of acute toxoplasmosis, atovaquone administration resulted in prolonged survival, with a reduction of parasite burdens in the blood and tissues during the course of treatment (Araujo *et al.*, 1991; Romand *et al.*, 1993). In a mouse model of chronic toxoplasmosis, atovaquone showed a decline in the number of tissue cysts and a decrease in the inflammatory response in the brain (Araujo *et al.*, 1991; Araujo *et al.*, 1992; Ferguson *et al.*, 1994). Indeed, atovaquone is effective against

reactivation, as it protected mice against reactivated toxoplasmic encephalitis (Dunay *et al.*, 2004).

In short term studies, buparvaquone efficiently inhibited the replication of *N. caninum* tachyzoites with an IC_{50} of 4.9 nM exerting parasiticidal activity after 9 days of culture in 0.5 µM or 6 days in 1 µM buparvaquone. However, in the long-term studies, the tachyzoites reached an adaption to high levels of buparvaquone. Additionally, ultrastructural changes confirm that buparvaquone acted rather slowly (Muller *et al.*, 2015a). In a non-pregnant mouse model of neosporosis, buparvaquone reduced the mortality and parasite load in the lungs, while the brain parasite load was higher than in untreated mice (Müller *et al.*, 2015a); the brain parasite load was lower when a 20-times reduced challenge dose was used (Müller *et al.*, 2016). In addition, in a pregnant mouse model of neosporosis, pup mortality and the transmission of *N. caninum* to offspring were strongly reduced by buparvaquone treatment of the dams (Müller *et al.*, 2016). In light of these results, atovaquone represents a valuable candidate to be tested for toxoplasmosis in ruminants and, although further studies are required to improve the efficacy in a mouse model, buparvaquone represents an obvious candidate to be tested against neosporosis in ruminants.

Anticancer agents

Another example of prospective compounds that provide interesting results is anticancer drugs, as parasites have several of the common characteristics of malignant tumours (Klinkert and Heussler, 2006; Dissous and Grevelding, 2011; Dissous and Grevelding, 2011), sharing a crucial feature of living and multiplying in a host organism. Tumour cells are defined by their independence from exogenous growth factors, their resistance to programmed cell death (apoptosis), and their infinite proliferative capacity. Unlimited proliferation and independence of growth factors are also characteristics of many parasites. Although it remains controversial whether apoptosis occurs in unicellular parasites (Debrabant *et al.*, 2003), it seems to be clear that intracellular parasites interfere with the programmed host cell apoptosis (Lüder *et al.*, 2001). Parasite and cancer cells disseminate in immune compromised tissues in order to escape host immune responses. Anticancer drugs may affect parasite survival at two completely different levels. Firstly, they might kill the parasite directly, if the target molecules of parasite and cancer cell are sufficiently similar. In this case, the original cancer drugs may serve as leader compounds and can be modified accordingly to specifically inhibit the parasite homologue. Secondly, to kill intracellular parasites successfully, the drug might also act on a host cell signalling pathway, which is essential for the parasite's survival. The advantage here is that the drug need not be modified, since it is already directed against the target molecule (Klinkert and Heussler, 2006).

Miltefosine (2-[hexadecoxy-oxido-phosphinoyl] oxyethyl-trimethyl-ammonium), an alkyl phospholipid and an analogue of the ubiquitous compound phosphatidyl choline found in eukaryotic cell membranes, was initially developed as an anticancer agent (Wieder *et al.*, 1999) and widely used for *Leishmania* therapy (Solano-Gallego *et al.*, 2011). It is highly active against extracellular *T. gondii* tachyzoites and exerts its activity by triggering apoptosis (Nyoman and Lüder, 2013). Miltefosine has no effect on a mouse model of acute toxoplasmosis after 5 days of treatment, but it shows activity in a 15-day treatment against chronic experimental toxoplasmosis, with a 78% reduction in the brain cyst burden. Pathological findings showed that the tissue cysts were smaller in size upon microscopical examination and that there were ultrastructural changes in the remaining cysts, suggesting that miltefosine effectively penetrates the blood-brain barrier (Eissa *et al.*, 2015). Against *N. caninum*, miltefosine showed an IC_{50} of 5.2 µM *in vitro* with a parasitostatic

effect at 25 µM for 10 hours and parasiticidal activity after 20 hours. In addition, *N. caninum* tachyzoites revealed ultrastructural changes after miltefosine exposure. In a mouse model of neosporosis, miltefosine improved mouse survival and reduced the cerebral parasite burden (Debache and Hemphill, 2012).

Recently, organometallic ruthenium complexes are object of great attention as antitumor agents with acceptable toxicity (Kostova, 2006; Bergamo and Sava, 2011). They also have antibacterial activity against some bacteria and parasites (Beckford *et al.*, 2011; Caroli *et al.*, 2012). Earlier studies indicated that ruthenium compounds interact with DNA (Schwietert and McCue, 1999), but more recent investigations showed that ruthenium compounds bind more strongly to proteins (Ravera *et al.*, 2004). Ruthenium compounds (compounds 16 and 18) were reported to exhibit IC_{50} values of 6–12 nM for *N.caninum* and 18-41 nM for *T.gondii* (Barna *et al.*, 2013). Furthermore, dinuclear thiolato-bridged arene ruthenium complexes (complex 9, complex 1 and complex 2) showed promising activities against *T. gondii* with IC_{50} values of 1.2 nM, 34 nM and 62 nM (Basto *et al.*, 2017). Therefore, anticancer drug such as miltefosine could be considered as an appropriate drug to be evaluated in ruminant model for toxoplasmosis and neosporosis whereas ruthenium complexes need to be evaluated in mice models before contemplate the option of testing in ruminants.

Endochin-like quinolones

Endochin is a 4-(1H)-quinolone initially investigated as an antimalarial drug (Salzer *et al.*, 1948). Subsequently, endochin was active against avian and murine toxoplasmosis (Gingrich and Darrow, 1951). Recent 4-(1H)-quinolone derivatives, endochin-like quinolones (ELQ), compounds that are analogs of ubiquinone, have been developed and tested against apicomplexan parasites such as *P. falciparum* and *T. gondii*, as well as other protozoa such as *Leishmania* parasites (Ortiz *et al.*, 2016). ELQs exert their activity by inhibit cytochrome c reduction by the cytochrome bc1 complex (Winter *et al.*, 2008; Doggett *et al.*, 2012). The cytochrome bc1 complex is a membrane-bound enzyme complex located in the inner mitochondrial membrane that contributes to pyrimidine biosynthesis and oxidative phosphorylation (Vercesi *et al.*, 1998). 3-alkyl-2-methyl-4(1*H*)-quinolones exhibited excellent *in vitro* activity against *P. falciparum* (Winter *et al.*, 2011). 4(1H)-quinolone-3-diarylethers, with improved stable and solubility properties, have been tested against *T. gondii* and ELQ-271 and ELQ-316 showed *in vitro* IC_{50} values of 0.1 nM and 0.007 nM, respectively. ELQ-271 and ELQ-316 were also efficacious against acute toxoplasmosis in mice when administered orally and against the cyst form of *T. gondii* in mice when administered intraperitoneally (Doggett *et al.*, 2012). Against *N. caninum*, ELQ-400 showed an IC_{50} value below 10 nM, had an impact on intracellular proliferation of tachyzoites and transmission electron microscopy showed that the primary target of ELQ-400 was the mitochondrion. In experimentally infected non-pregnant mice, ELQ-400 orally showed a reduction in the number of animals with lung and brain infection, as well as a reduction in the humoral immune response against *N. caninum* (Müller *et al.*, 2017a). Thus, ELQ-316 is a promising starting point for the development of a future toxoplasmosis therapy in ruminants, and ELQ-400 showed hopeful results against *N. caninum*, but further studies are needed to assess efficacy in pregnant animal models.

Calcium-dependent protein kinase inhibitors

Calcium signalling is a very important pathway that regulates diverse cellular processes (Berridge *et al.*, 2000). In apicomplexan parasites, this signalling pathway directs motility, cell invasion and egression (Lourido *et al.*, 2012). Members of the family of calcium dependent protein kinases (CDPK's) are abundant in certain pathogenic parasites and absent in mammalian

cells making them strong drug target candidates. Genetic disruption of CDPKs has shown they control a wide range of phenotypes in *T. gondii* including egress (TgCDPK1 and TgCDPK3) (Lourido *et al.*, 2010; Garrison *et al.*, 2012; Lourido *et al.*, 2012; McCoy *et al.*, 2012), microneme secretion (TgCDPK1) (Lourido *et al.*, 2012), motility (TgCDPK1) (Lourido *et al.*, 2010), or cell division (TgCDPK7) (Morlon-Guyot *et al.*, 2014). Other CDPKs in *T. gondii* (CDPK4, CDPK4A, CDPK5, CDPK6, CDPK8, and CDPK9) were described non essential for the lytic cycle (Wang *et al.*, 2016). CDPKs may are also involved in other functions in *T. gondii*, for example, CDPK2 is essential for bradyzoite development (Uboldi *et al.*, 2015) and CDPK4A, CDPK6 and CDPK7A may play roles during sporozoite formation or transmission via oocysts (Long *et al.*, 2016).

Bumped kinase inhibitors (BKIs), a particular class of pyrazolopyrimidine inhibitors of CDPK1, have bulky C3 aryl moieties entering a hydrophobic pocket in the ATP binding site. BKIs selectively inhibit CDPK1 from apicomplexans in a good structure-activity relationship (Keyloun *et al.*, 2014), but they do not inhibit mammalian kinases because they have larger amino acid residues adjacent to the hydrophobic pocket, thereby blocking the entry of the bulky C3 aryl group. CDPK1 is found in most apicomplexans, and the highly conserved nature of the ATP binding domain shared by apicomplexan CDPK homologues could be exploited, to some extent, in the development of potential broad-spectrum inhibitors (Keyloun *et al.*, 2014). BKIs were originally developed to combat malaria (Ojo *et al.*, 2012), but they have been tested for many apicomplexan parasites (Van Voorhis *et al.*, 2017). BKI-1294 acted with an IC_{50} of 20-220 nM *in vitro* for different strains of *T. gondii* (Winzer *et al.*, 2015). BKI-1294 was highly effective against acute toxoplasmosis in mice, decreasing the numbers of *T. gondii* tachyzoites in the peritoneal lavage fluid (Doggett *et al.*, 2014) and in a murine vertical transmission model of *T. gondii (Müller et al., 2017)*.

BKI-1517 showed a 3-times lower IC_{50} than BKI-1553 and BKI-1294 against *N. caninum in vitro* (Ojo *et al.*, 2014; Müller *et al.*, 2017b). In a pregnant mouse model, BKI-1294, BKI-1517 and, less clearly, BKI-1553 achieved protection against the vertical transmission of *N. caninum*, but BKI-1553 and more markedly BKI-1517 showed detrimental effects on fertility (Winzer *et al.*, 2015; Müller *et al.*, 2017b). Hence, BKI-1294 could be considered a promising drug to be tested in ruminant models for toxoplasmosis and neosporosis.

Since BKIs showed parasitostatic rather than parasiticidal effects, they are appropriated for a combined immunization plus treatment protocol. These compounds triggered the formation of relatively long-lived multinucleated complexes where parasites are blocked in the process of cytokinesis and remain trapped within the host cell but are still viable. These multinucleated complexes express specific antigen SAG1 and the bradyzoite marker BAG1 (Winzer *et al.*, 2015; Müller *et al.*, 2017b). Thereby, these antigens may be presented to the immune system eliciting stable immune responses against tachyzoite as well as bradyzoite stages.

Apart from CDPK1, the rarity of kinases containing small gatekeeper residues in the apicomplexan genome reduces the chance of off-target effects, although intermediate sensitivity is expected for kinases containing A, S, or T in the gatekeeper position, thus potentially limiting this approach in some cases. However, an interesting strategy based on the exploitation of the kinase gatekeeper residue was recently developed to identify parasite CDPKs substrates (Lourido *et al.*, 2013).

Conclusions

Although vaccination has been considered the better control option for ovine and caprine toxoplasmosis and bovine neosporosis, drug therapy should be a viable approach, possibly when combined in an immune-chemotherapy strategy. A large number of experimental efficacy drug studies against *T. gondii* and *N. caninum* infections has been carried out so far in mouse models, but studies in farm ruminants are scarce, showing a lack of efficacy. Regarding toxoplasmosis, several drug treatments that were applied before (spiramycin or sulphonamides and pyrimethamine) or after infection (monensin or decoquinate) have been carried out in pregnant ewes to reduce the outcome of ExTT after experimental infection of the ewes at the mid-stage of pregnancy. Sulphonamides are also useful in field conditions to reduce *T. gondii*-related abortions, and toltrazuril is applied to chronically infected lambs to reduce tissue cysts. Concerning neosporosis, the triazinones administered in infected newborn calves or lambs modulate the humoral immune response and reduce the clinical signs and *N. caninum* detection in organs. In addition, triazinones, along with folate inhibitors, can reduce abortions due to neosporosis under natural conditions. Finally, monensin seems to reduce the humoral immune response in non-pregnant cattle, and decoquinate could decrease abortions and transplacental transmission in chronically and primo-infected pregnant heifers. Despite these efforts, no safe and effective drug is available for toxoplasmosis and neosporosis in farm ruminants at present, so new approaches in drug development are needed. These new developments should also focus on the therapy of toxoplasmosis in humans. Highly promising future drugs against *T. gondii* and *N. caninum* have been tested *in vitro* and in small animal models; most of them target tachyzoites efficiently, but they are missing information regarding their efficacy against the bradyzoite stage. Best results have been reported with artemiside, atovaquone, miltefosine, ELQ-316 and BKI-1294 against *T. gondii* and with DB745, buparvaquone, miltefosine, ELQ-400 and BKI-1294 against *N. caninum*.

Conflic of interest

The authors declare that they have no conflicts of interest. This work was supported by the National Institutes of Child Health and Human Development (NICHD, grants R01 AI 111341 and R01 HD 080670), the United States Department of Agriculture (USDA, grant 2014-67015-22106), the Community of Madrid (PLATESA grant No. S2013/ABI2906) and the Iberoamerican Science and Technology Programme for Development (CYTED, Grant No. 113RT0469). Roberto Sánchez Sánchez was financially supported through a grant from the Spanish Ministry of Education, Culture and Sports (grant No. FPU13/03438). Patricia Vázquez was supported by the postdoctoral Juan de la Cierva-Formación program of the Spanish Ministry of Economy and Competitiveness (grant No. FJCI-2014-20982).

Acknowledgements

Roberto Sánchez Sánchez is supported by a fellowship from the Spanish Ministry of Education, Culture and Sports (MECD), as a part of the Program of Training of University Teaching Staff (FPU, grant number FPU13/03438). Patricia Vázquez has a Juan de la Cierva-Formación post-doctoral contract (FJCI-2014-20982) from the Spanish Ministry of Economy and Competitiveness (MINECO). Also, Public Health Service, National Institutes of Health, Bethesda, MD (grants R01 AI 111341 and R01 HD 080670), the U.S. Department of Agriculture (grant 2014-67015-22106) and the Community of Madrid, Spain (PLATESA, S2013/ABI2906) are acknowledged for their financial support.

"El éxito es la habilidad de ir de fracaso en fracaso sin perder el entusiasmo"
Winston Churchill (1847-1965)